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PECAN LEAF BLOTCH [1]

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INTRODUCTION

Pecan leaf blotch caused by the fungus *Mycosphaerella dendroides* (Cke.) comb. nov. was first collected by the senior writer near Rockport, Ind., in 1923. The following year it was observed at Thomasville, Ga., but at that time it was found only occasionally and was not considered economically important. No extensive survey to determine the exact distribution of the disease has been attempted, but it is now known to occur in many pecan orchards and nurseries in Georgia, Florida, Alabama, Mississippi, and Louisiana. The writers' observations lead them to believe that it is rapidly increasing in importance and is gradually extending its range.

THE DISEASE

Leaf blotch is first found in June on a few of the older leaves of the pecan and may gradually increase until all mature leaves are spotted or killed. On nursery trees it first appears on the lower and older leaves, but as the season advances the others are also attacked. Defoliation progresses upward, till by the 1st of November only a few of the youngest and uppermost leaves remain.

Injuries caused by borers, rosette, general neglect, or any other factor that lowers the vitality of trees predispose them to attack by the leaf-blotch fungus. As is usual with a foliage disease, the damage caused by it can not be estimated accurately. The presence of other leaf-spot fungi, such as *Cercosporella caryigenum* (E. and E.) v. Hohnel, *Cercospora fusca* Rand, *Cladosporium effusum* (Wint.) Demaree, and *Phyllosticta caryae* Pk., increases the difficulty of estimating losses. The loss, however, at times is considerable, judged by the extent of the defoliation.

Trees too thickly planted or growing in land that receives no cultivation often lose one-half to three-fourths of their leaves prior to the harvest season on account of leaf blotch. Such heavy defoliation results in nuts of inferior quality and a light crop the year following. Pecan nurseries seem to furnish excellent conditions for infection and for propagation of the fungus. The severity of the attack on nursery-tree foliage may not be due so much to the crowded condition of the trees as to the presence of innumerable infection sources on the ground beneath them. Since cultivation of the nursery ordinarily is

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not deep enough to cover the previous year's infected leaves, many are left either uncovered or only partially covered, and in these the fungus lives through the winter. Trees growing in good soil, well fertilized and cultivated, are not attacked with the same severity as neglected trees.

The diseased areas show on the upper surface of the leaf first as undefined faint yellow spots which gradually change to dark brown without any clear line of demarcation. The spots on the lower surface are more distinct as they are occupied by a dense growth of erect conidia and conidiophores. (Fig. 1, A.) Later this aerial growth collapses and forms a layer that completely hides the affected host tissues. In mass the color of the aerial fungous structure is at first dark brown, but later, after becoming matted, it appears greenish brown. The spots vary greatly in size, from 1 to 8 mm. in diameter. They may be few in number, but more frequently they are quite numerous, crowded, and coalesce, forming large blotches that may involve a large portion or even the entire lower leaf surface.

During midsummer, black conical pycnidia commonly form on the affected host tissues underneath the mats of conidia and conidiophores. As the season advances the pycnidia increase in number and area and often extend beyond the limits occupied by the aerial hyphae and conidia. Primordia of perithecia develop among the pycnidia during early autumn. The latter structures are not at first easily distinguished from the pycnidia. During the course of the summer the conidia and conidiophores are washed away by rains, thus exposing to view the more conspicuous black pycnidia and immature perithecia. (Fig. 1, B.)

THE CAUSAL FUNGUS

MORPHOLOGY

Conidial Stage

The conidiophores penetrate the lower epidermis either singly or in small clusters and irrespective of the stomatal openings. They are light to dark brown, erect or decumbent, and may be either simple or branched. (Fig. 2, A and B.) The first-formed conidia are produced near the apical end of conidiophores originating from subepidermal hyphae. Later decumbent hyphae grow over the spots and also bear conidia.

As a rule the conidia are formed singly, but sometimes they are found in short chains of two to three. (Fig. 2, C.) They are subhyaline to light brown, elongate, slightly curved, and 8 to 10 septate; and each segment almost invariably has a large and conspicuous vacuole. The greatest thickness of a conidium is at a point about one-fourth to one-third of its length from the basal end. From the thickest part a conidium tapers toward both ends, but the apical end is drawn out to a more or less attenuated prolongation, giving the conidium a pronounced rostrate appearance. All segments of the conidia, even the hyaline apical ones, may produce germ tubes. (Fig. 2, E.)

Pycnidial Stage

As stated previously, small black structures are formed during the early development of the spots on the underside of pecan leaflets. These bodies were first considered as spermagonia, but since there

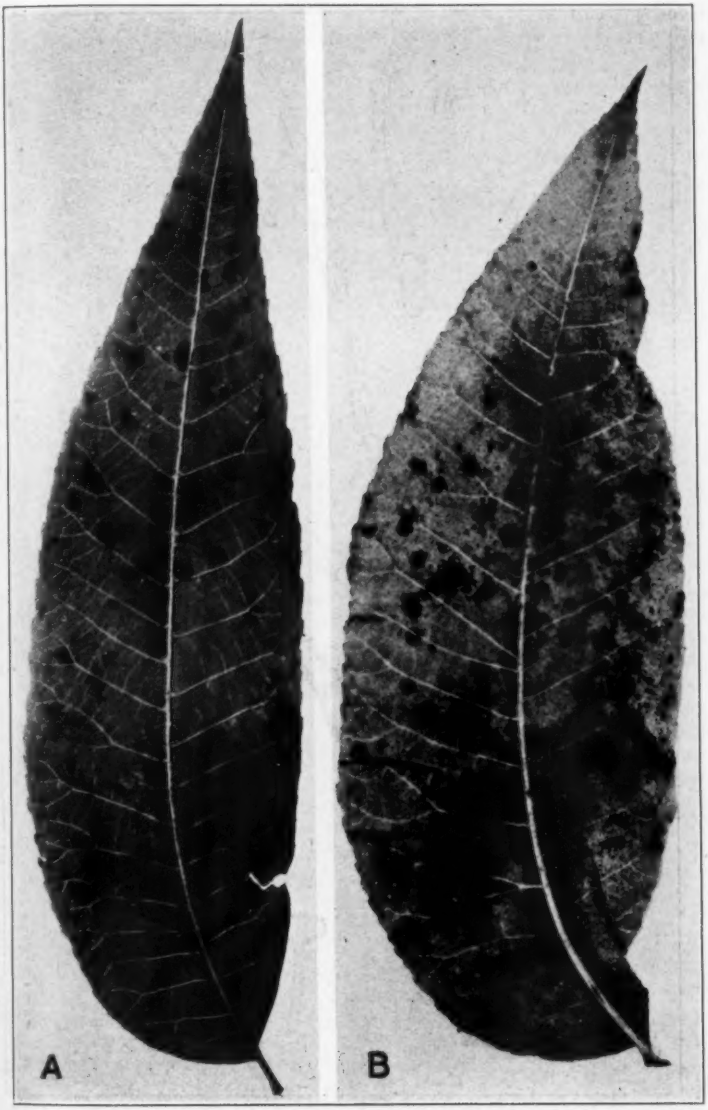

FIGURE 1.—Different stages of pecan leaf blotch on the undersides of leaflets: A, Early stage, showing spots caused by groups of conidia and conidiophores; B, later stage, showing blotched effect caused by groups of pycnidia and immature perithecia

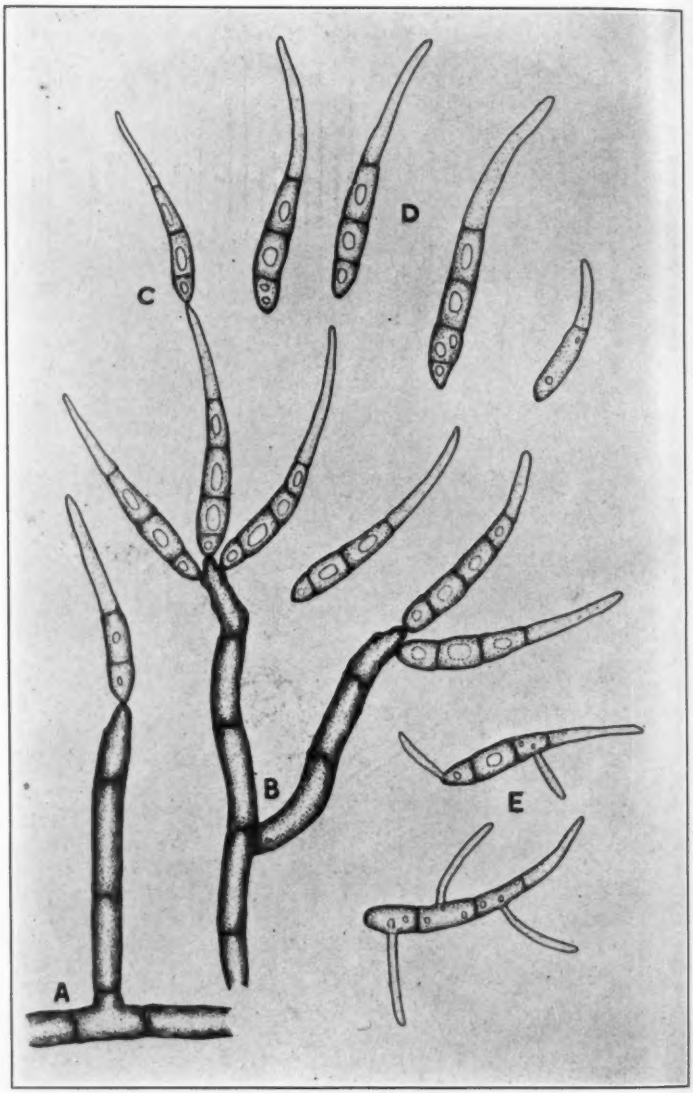

FIGURE 2.—Conidia and conidiophores of the pecan leaf-blotch fungus, *Mycosphaerella dendroides*: A, Conidiophore growing from decumbent hyphae; B, branched conidiophores originating from subepidermal hyphae; C, catenulate conidia; D, representative conidia, showing different shapes and sizes; E, germinating conidia. × 825

is no proof of their functioning as such, it is thought best to refer to them as pycnidia. This spore form may be the same as *Phyllosticta convexula* described by Bubak (*1*).[3] The spores are of about the same size, and Bubak reported that his Phyllosticta was associated with immature perithecia of a Mycosphaerella.

The pycnidia, which are at first brown and subepidermal, later break through the epidermis and become black. About one-half to two-thirds of the pycnidium is embedded within the host tissue, out of which it can readily be lifted with a sharp-pointed needle. The pycnidial wall is two to four cells thick. The contents of the pycnidia are composed of many small rod-shaped bodies, 0.5 to 1μ across and 2 to 4μ long. (Fig. 3, A.) Repeated attempts to germinate the pycnospores in tap water, distilled water, or in poured plates of various culture media have been uniformly unsuccessful.

The pycnidia mature shortly after they emerge through the epidermis, and irregular cracks or lacerate openings appear in the apical part, thus allowing the contents to emerge as a small waxy mass. Microtome sections of old pycnidia show that many of them are either partially or entirely empty. Conidiophores commonly grow from the exposed parts of the pycnidia, and sections show the basal end of the conidiophores attached to the pycnidial wall.

PERITHECIAL STAGE

The perithecia develop either singly or in groups, principally on the undersides of the green leaves during the latter half of the summer, but they do not mature until the following spring. The groups are circular to irregular in outline and often conform to the shape of the spaces between the leaflet veins. (Fig. 3, B.) A mixture of pycnidia and perithecia often occurs. Almost invariably conidia and conidiophores are found intimately associated with the immature perithecia. In fact, conidiophores often arise from perithecia, and microtome sections show them to be connected with the perithecial body. Young perithecia can not always be distinguished macroscopically from pycnidia, nor always by the aid of a hand lens or a binocular microscope. Ordinarily, however, they are larger, more irregular in form, and frequently have a flat or convex top while immature. Before rupturing the cuticle, and for some time afterwards, they have a shiny appearance. Finally they become dull and rough.

Perithecia at first are embedded within the host tissues, but later they become erumpent. Microtome sections exhibit the very young perithecia as small masses of short thin-walled hyaline cells with no perithecial wall. (Fig. 3, E.) The perithecial wall first begins to form at the apex of the undifferentiated mass which lies next to the lower epidermis of the leaf. (Fig. 3, F.) The cells of the wall form successively toward the base of the young perithecium and finally inclose the mass. The perithecium soon reaches its full size, ruptures the epidermis, and is plainly seen with the naked eye.

A well-developed ostiole is formed, but until the ascospores are mature it is closed by a layer or plug of fungous tissue. (Fig. 4.) As the ascospores approach maturity, the consequent increase in pressure of the asci upon the plug results in the formation of an opening through which the ascospores are ejected, often with force sufficient to

[3] Reference is made by number (italic) to "Literature cited," p. 789.

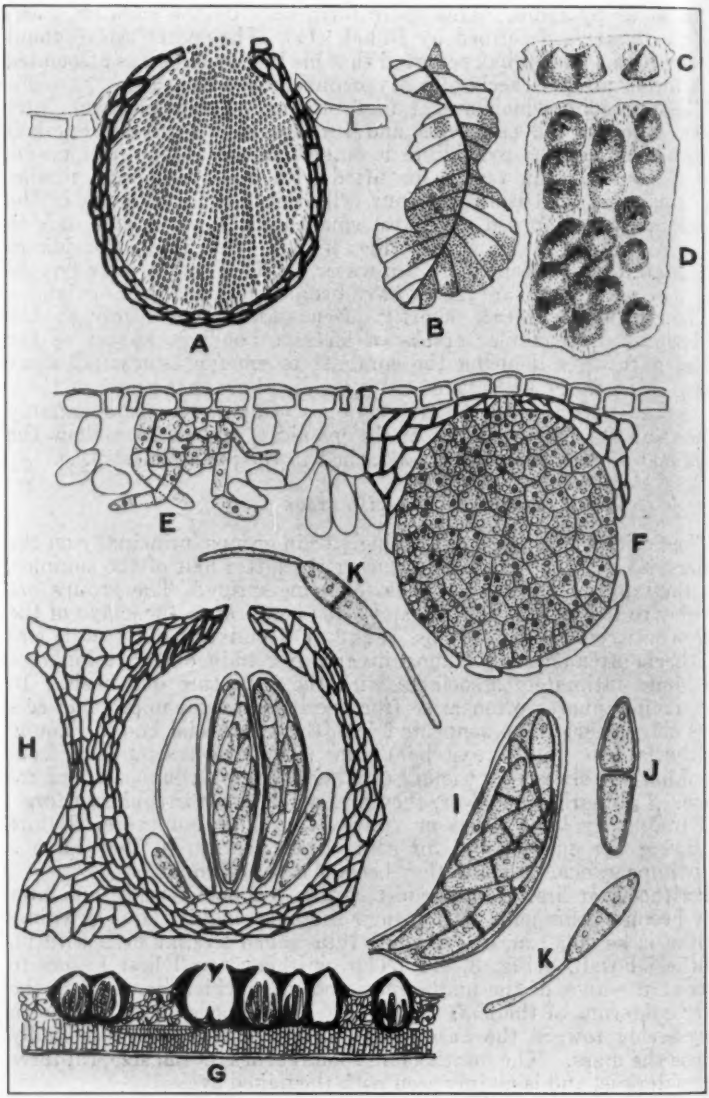

FIGURE 3.—Pycnidial and perfect stages of the pecan leaf-blotch fungus, *Mycosphaerella dendroides*: A, Pycnidium containing spores. × 430. B, Old pecan leaflet with mature perithecia. × ½. C, D, Enlarged perithecia. × 60. E, Primordium of perithecium. × 450. F, Immature perithecium, showing character of contents and early development of perithecial wall. × 450. G, Cross section of perithecia, indicating their position in relation to host tissues. × 70. H, Cross section of a mature perithecium. × 450. I, Representative ascus, showing typical shape and thickened apex of wall. × 960. J, Typical shape of spores. × 1,125. K, Germinating spores. × 770

throw them 3 to 5 mm. Although the development of the perithecia begins within the tissues of the green leaves shortly after the appearance of the conidial stage, the asci do not ordinarily mature before late winter or early spring. Attempts to hasten the maturity of the perithecia and the formation of the ascospores by placing the infected leaves in a moist chamber in the laboratory have proved unsuccessful, except in one case when mature asci were produced in the laboratory in January.

The asci are hyaline, cylindrical clavate, and are not accompanied by paraphyses. (Fig. 3, G and I.) The ascospores are hyaline and 1-septate, each spore being constricted into two slightly unequal cells. The cell which is pointed toward the base of the ascus has a blunt end; the other cell is thickest at a point near the septum and then tapers gradually. (Fig. 3, J.) The method by which the spores are liberated from the asci is not definitely known. The fact that spores may be collected by inverting a plate of agar over leaf tissues containing perithecia indicates that they are ejected through the ostiole with some force.

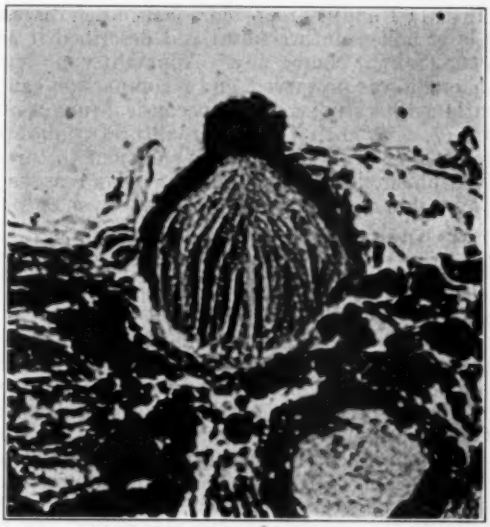

FIGURE 4.—Photomicrograph of an immature perithecium of *Mycosphaerella dendroides*. × 1,500

The relationship of the conidial and the perithecial stage is shown by their constant association, by the development of the conidiophores from hyphae forming part of the perithecial wall, and by the production of similar conidia in artificial cultures from monospore cultures of ascospores. The genetic relation of the pycnidia to the other two spore forms is shown by their regular appearance on the same lesions with the conidiophores and perithecia and by their formation in cultures made from either conidia or ascospores.

IDENTITY OF THE ORGANISM

In 1890 B. D. Halsted collected a fungus on hickory leaves near Cold Spring, N. Y. Later Ellis and Everhart (*3*) described this fungus as *Cercospora halstedii*. The writers have not been able to find any other reference in literature to this fungus except one made by Fawcett (*4*) in 1909 and one by Waite (*10, p. 188*) in 1911. Fawcett referred to a disease of pecan leaves in Florida which "produces large brown or black blotches from one-fourth to three-fourths inch in size and irregular in outline," but he did not describe the causal fungus. From the meager discussion one can not be certain

whether Fawcett was dealing with *C. halstedii* or with *C. fusca* Rand, a widely distributed fungus which causes circular to irregular brown spots on pecan leaves. *C. fusca* was first described by Heald and Wolf (*5*) in 1911 as *Clasterosporium diffusum*, but the name was later changed by Rand (*7*) to *Cercospora fusca*.

On several occasions the writers have collected near Thomasville, Ga., on hickory leaves, a fungus which is considered by W. W. Diehl as *Cercospora halstedii*. In all essentials the conidial stage of the fungus causing the pecan-leaf blotch reported in this paper is the same as *C. halstedii* found on hickories in Georgia, and the two forms are considered by the writers as identical. While both conidia and pycnospores taken from hickory and pecan leaves have been compared, mature perithecia have not been observed on hickory specimens collected in southern Georgia.

In 1911 Rand (*6*) reported a leaf-blotch disease of the pecan (which he later called anthracnose) and described it as *Mycosphaerella convexula* (Schw.) comb. nov. Apparently no specimen of his Mycosphaerella was preserved, and a comparison can not be made. Rand continued his studies of this pecan-leaf fungus and reported two years later (*7, p. 319*) that "further cultural studies of the fungus brought out changes in its morphology sufficient to throw it out of the genus Mycosphaerella." He summarized his reasons for changing the determination of the fungus as follows (*7, p. 329*):

> From the general pathology and temperature relations, the cross-inoculations and cultural studies, and finally from the morphology of the pecan fungus there can be no doubt of its specific connection with *Glomerella cingulata* (Stonem.) S. and v. S., the fungus causing bitter-rot of apples, ripe-rot of grapes, and anthracnoses of a wide range of hosts.

The outstanding facts brought out in that paper which seemed to have influenced Rand to reclassify the fungus were that the ascospores gradually changed in cultures from a 1-septate to a unicellular form, that the diseased areas on the leaves and cultures regularly produced a Gloeosporium type of conidia, and that the fungus when inoculated in apples produced a decay typical of apple bitter rot.

Rand's later report seems to preclude any reason for considering the Mycosphaerella reported in his earlier publication as being related to the one described in this paper. In 1927 the writers [4] provisionally referred the pecan leaf-blotch fungus to *Mycosphaerella* (*Sphaeria*) *convexula* Schw., but after making a more critical study of the type specimen of that fungus they concluded that, on account of morphological differences between the two fungi, the reference was not tenable.

In 1822 Schweinitz (*8*) described a fungus found on Quercus as *Sphaeria dendroides*. Later, however, he published a note (*9*) which indicated that he regarded *S. dendroides* as a synonym of *S. myriadea* Fr. Seemingly the next reference to this specific name in literature was made by Cooke (*2, p. 108*), who described a fungus found on Carya as *Sphaerella dendroides* Schw. Cooke mentioned that "this species has been confounded with *Sphaeria myriadea* from which it is evidently distinct." Cooke's description is apparently based on specimens issued by Ravenel in his Fungi Caroliniani as No. 61.

[4] DEMAREE, J. B., and COLE, J. R. TWO UNREPORTED LEAF SPOTS OF PECANS. U. S. Dept. Agr., Bur. Plant Indus. Plant Disease Rptr. 11: 135-136. 1927. [Mimeographed.]

The specific name that he used was that of Schweinitz in a new combination, the original name having been applied by Schweinitz to a fungus on oak, doubtless correctly referred to as *Sphaeria myriadea* Fr. It seems, therefore, that the name *Sphaerella dendroides* is really not that of Schweinitz, but should be quoted as *S. dendroides* Cke.

Morphologically, the perithecial stage of the pecan leaf blotch and *Sphaerella dendroides* as described by Cooke (*2*) appear to be identical. The size and shape of the ascospores correspond quite closely. Cooke stated in his description that the ascospores of *S. dendroides* were 4μ wide and 23 to 24μ long. Since the name Mycosphaerella is in better usage than Sphaerella and is now employed to designate fungi forming perithecia and spores resembling those described in this paper, the writers suggest that the pecan leaf-blotch fungus be regarded as *Mycosphaerella dendroides* (Cke.) comb. nov.

EMENDED DESCRIPTION

Mycosphaerella dendroides (Cke.) comb. nov. *Sphaerella dendroides* Cke. *2, p. 108*), not *Sphaeria dendroides* Schw. (*8, p. 47*).

Perithecia hypophyllous at first, subepidermal, finally erumpent, single or crowded, aggregate in irregular groups, or more or less over the entire lower leaf surface; globose to irregularly subglobose, 75 to 125μ in diameter, shiny black, later roughened. Perithecial walls usually four to six cells thick, thicker around ostiole. Asci clavate to cylindrical, thin walled, thickened at apex, hyaline, 38 to 66μ by 10 to 15μ. Ascospores 18 to 26μ by 4 to 6μ.

Pycnidia erumpent, black, globose to spherical, conical, 75 to 90μ wide. Pycnospores hyaline, nonseptate, 2 to 4μ by 0.5 to 1μ.

Conidia subhyaline to light brown, elongate, subclavate, rostrate, terminal or lateral, solitary or in chains of two to three, 35 to 83μ by 4.5 to 7μ, 1-septate to 8-septate, usually with a conspicuous vacuole within each segment. Apical segment long and attenuate. Conidiophores erect to prostate, simple or branched, light to dark brown, emerging either singly or in groups of two to eight. Hyphae intercellular within the tissue of mature leaves, subhyaline, branched, and septate.

The fungus is parasitic in mature leaves of Hicoria species. The perithecia first appear on green leaves during the latter part of the growing season and mature on fallen leaves the following spring. The perithecial stage has also been reported on leaves of Hicoria from South Carolina.

PHYSIOLOGY

Growth on Artificial Media

Some difficulty was at first experienced in obtaining cultures from either conidia or ascospores. Only about 10 per cent of the conidia and a smaller percentage of the ascospores have been induced to germinate in the laboratory. The early growth is very slow, the cultures increasing about 50μ in diameter a day for the first 10 days or 2 weeks when grown on corn-meal agar and Lima-bean agar.

Single-spore cultures of the conidial stage of the fungus were obtained in the usual way. Ascospores used for culture work were obtained by catching spores in a plate of agar inverted about 2 to 3 mm. above sections of leaves bearing mature ascospores and lying on wet filter paper. Germination and growth were followed under the microscope, and isolated germinating spores were marked and transferred to another plate of agar. When the cultures became macroscopic in size they were transferred to agar slants.

Monospore cultures require a period of 10 to 12 days under optimum temperature conditions to produce macroscopic growth. The media

used were of three types—nutrient agars, vegetable plugs, and nutrient liquids. Of the nutrient agars, the fungus grew best on those made from Lima beans or dextrose, attaining a maximum growth of 10 mm. in diameter in 60 days. In these two agars the fungus formed a black stroma, slightly crinkled, mostly submerged, and covered with an abundance of white mycelium. On prune agar the fungus formed a black stroma, raised and crinkled, but produced no aerial mycelium. Of the vegetable plugs used, the greatest growth was on sweetpotato. On this medium the stroma was crinkled, elevated, black, and covered with short, grayish mycelium. In the liquid, i. e., corn-meal and nutrient solution, the growth was slight and consisted of a "floating stroma" about 8 mm. in diameter, black and covered with short hyphae.

Conidia are commonly produced in young cultures growing upon media rich in carbohydrates and appear to arise from vegetative hyphae rather than from conidiophores, which are not produced in artificial cultures. The conidia are slightly abnormal and do not always have the regular shape and the same number of septa as those found on the host.

Pycnidia containing the characteristic small rod-shaped pycnospores found on pecan leaves are produced in abundance in both old and new cultures. Although cultures of this fungus have been under observation for three years, no evidences of the presence of perithecia have been noted.

Temperature and pH Relations

The temperature for the growth of this organism ranges from 16° to 31° C. The optimum is 27° and the lethal is about 36°. The fungus makes little or no growth when subjected to a temperature of 16° or below. In the studies of the fungus with respect to hydrogen-ion concentration, corn-meal agar with a pH reading ranging from 3.5 to 9.7 was used. The cultures were held at the optimum temperature (27°) for 60 days before the final reading was made. Sparse growth occurred at pH 3.5 to 5.2 without production of aerial mycelium. The maximum growth was made at pH 7.5. The color of the aerial mycelium on the stroma in the hydrogen series ranged from olive green to dark brown.

Pathogenicity

The exact conditions that favor germination and infection have not been demonstrated. Infections have never been known to occur on young pecan leaves and have been found on full-grown ones only after they have assumed the dark-green color. The writers do not know whether this indicates that young leaves are immune or that the incubation period extends over a relatively long period. Repeated attempts to infect the leaves of potted seedlings in the laboratory and the leaves of trees in orchards and nurseries by means of conidia and ascospores have been unsuccessful. When the work was done in the laboratory the inoculated plants were covered with a bell jar for 24 to 48 hours, and the inoculated leaves of larger trees outdoors were inclosed in either glassine bags or celluloid chambers for 24 hours.

Since artificial inoculations with both conidia and ascospores have not resulted in infection, it has not been possible to comply with Koch's rules; but the production of conidiophores and conidia on

living leaves in areas at first nonchlorotic shows beyond doubt the connection of the conidial stage of the fungus with the disease. The presence of the fungus itself is one of the first signs of the disease.

CONTROL

Orchard and nursery sanitation will undoubtedly play an important part in the control of the pecan leaf blotch. Cultural practices for effecting orchard sanitation, now coming into general use in the control of pecan scab, will undoubtedly have to be extended as aids in the control of other pecan diseases. The leaf-blotch fungus is not known to attack any part of the pecan tree except the leaf blades. As the fungus overwinters only on these parts, it is reasonable to assume that if the leaves are plowed under during late winter or early spring infection of the new crop of leaves will be materially lessened.

The writers have observed that blotch is almost absent in orchards where spray or dust was used against pecan scab. Three applications of Bordeaux mixture or four or five applications of monohydrated copper sulphate and lime dust have uniformly reduced blotch to a negligible factor.

During the season of 1928 a preliminary experiment was made to test the efficacy of a dust composed of 20 per cent monohydrated copper sulphate and 80 per cent hydrated lime as a control for pecan leaf blotch in nurseries. The work was done near Cairo, Ga., in a block of 3-year-old nursery trees, which was divided into five plots and treated on the following dates: Plot 1, May 11 and 31, June 22, July 16 and 30; plot 2, May 31, June 22, July 16 and 30; plot 3, June 22, July 16 and 30; plot 4, July 16 and 30; and plot 5, July 30.

The control of the disease in plots 1, 2, and 3 was excellent, and only a slight difference could be noticed among them. Considerable blotch was present, however, in plots 4 and 5. A comparison of A and B of Figure 5 shows clearly what was accomplished with five applications of dust.

The results of the test brought out two important facts. They showed that under the conditions of the experiment the disease was readily controlled with the copper-lime dust and they indicated that the June and July applications were the most important and that earlier treatments may not be necessary to effect commercial control.

SUMMARY

Leaf blotch, a disease of pecan leaves caused by the fungus *Mycosphaerella dendroides* (Cke.) comb. nov., is known to occur in Georgia, Florida, Alabama, Mississippi, Louisiana, and Indiana. The disease is rapidly increasing in importance and seems to be extending its range of distribution. It is also known to occur in South Carolina and Georgia on other species of Hicoria.

The conidial stage of the fungus which attacks mature leaves of the host during the latter half of the season is here considered identical with *Cercospora halstedii* E. and E. The fungus is different from *C. fusca*, a widely distributed pecan parasite.

The most conspicuous sign of the disease is the presence of groups and blotches of pycnidia and immature perithecia on the undersides

of the leaf blades. The asci mature on the fallen leaves during the spring.

The morphological characters of the conidial, pycnidial, and ascogenous stages are described.

FIGURE 5.—A, Untreated plot, showing defoliation of nursery pecan trees by the leaf-blotch fungus; B, plot dusted five times with a mixture of 20 per cent monohydrated copper sulphate and 80 per cent hydrated lime. Both plots photographed November 16, 1928

The relationship of conidial and perithecial stages is indicated by both stages being found on the same lesions, by the similarity of cultures from either conidia or ascospores, and by the production of similar conidia and pycnidia in artificial cultures made from either conidia or ascospores.

Orchard sanitation will undoubtedly play an important part in the control of the disease. A preliminary experiment indicated that monohydrated copper sulphate and lime dust will effectively control the disease and that midsummer treatments are the most important.

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CELL-SAP CONCENTRATION AND TRANSPIRATION AS RELATED TO AGE AND DEVELOPMENT OF COTTON LEAVES[1]

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INTRODUCTION

This paper deals with the interrelations between the concentration of cell sap and the rate of transpiration of cotton leaves as influenced by age, and the relation of these factors to leaf development.

The experimental plants were of the Pima variety of Egyptian cotton grown as a part of a water-requirement series at Sacaton, Ariz., in 1927. During the growth of the plants all lateral buds were removed as they appeared. Limiting aerial growth to the main axes and leaves resulted in vigorous plants provided with an unusual number of leaves on the main stalk. The date of appearance of each of the leaves was recorded throughout the summer.

No procedure for the direct determination of the individual transpiration rates of attached leaves fully exposed to the weather has yet been devised. It is possible, however, to obtain relative values by the leaf-temperature method, as measurements already reported (*5*)[3] have shown that the differences in the temperatures of similarly exposed cotton leaves are inversely proportional to the differences in their transpiration rates. The correlation found between the hourly differences in the temperatures and transpiration rates of the leaves of plants in dry and moist soils was -0.93 ± 0.025. This inverse relationship between transpiration and leaf temperature is graphically illustrated in Figure 1.

Weighing is the most direct method of studying transpiration, and where whole plants are used it yields the most reliable information. Many investigators, however, have used the method of comparing the transpiration rates of cut leaves. Deductions from such measurements are of uncertain value, since it can not be assumed that they are applicable to attached leaves on rooted plants. Palladin (*15, p. 138*) cited Krutizky to the effect "that a single leaf of *Cyssus antarcticus* lost 10.6 c. c. of water in one day, while a branch of the same plant with six leaves lost but 10.8 c. c." Hygrometric paper has been used in studying transpiration, and the method has certain advantages over direct measurements in that it can be used on attached leaves. The hygrometric-paper method is tedious and requires careful comparisons with standard colors, in which comparisons time and temperature are both factors of some significance. The great disadvantage of this method is that it does not measure the normal transpiration rate of a leaf but gives only an index to what is termed the "transpiring power."

[1] Received for publication Aug. 10, 1929; issued May, 1930.

[2] The photographs for Figures 2, 3, and 5 were taken by H. F. Loomis, Assistant Agronomist, Office of Cotton, Rubber, and Other Tropical Plants.

[3] Reference is made by number (italic) to "Literature cited," p. 802.

The portion of the leaf studied is covered with the paper and a glass slide during the observation.

The leaf-temperature method is not only convenient and applicable to attached leaves, but also it has a further advantage in that it is rapid. The leaves, until the moment of a measurement, are fully exposed to the weather, and during the few seconds required for a reading by the procedure employed there is little opportunity for a material change in leaf temperature. The high correlation found between transpiration rates and leaf temperatures suggested the possibility of using the method to secure the measurements and comparisons here reported.

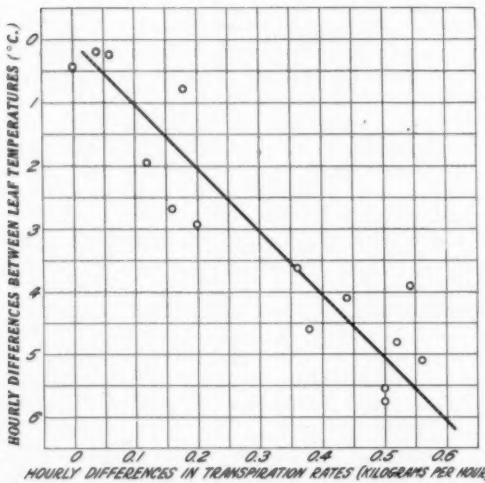

FIGURE 1.—Relation between hourly leaf-temperature differences (wilted minus turgid) and the corresponding hourly differences in transpiration rates (turgid minus wilted) of cotton plants grown in dry and in moist soils. The transpiration values are each for a full hour, whereas the leaf temperatures were measured over a much shorter period at the middle of the hour. Fluctuations in environmental factors quickly influence leaf temperatures as they do transpiration rates, but such fluctuations may make only a minor impression on the total transpiration of an hour. These measurements, which cover one day, have been shown (*5*) as an hour graph of the two variables with respect to time

REVIEW OF LITERATURE

An examination of the literature dealing with the sap concentration and the transpiration rate of leaves of different ages brings out the fact that investigators, using various methods, have found great variability in the directions that these gradients take in different plants.

Schechner[4] reported that the transpiration of young leaves of many plants was greater than that of older leaves and in some plants leaves of intermediate age had lower transpiration rates than either older or younger ones. Fleischmann and Hirzel,[4] on the contrary, found equal surfaces of old hop leaves to transpire 2.5 times as much water as young leaves. Seeliger,[4] working with cut leaves from woody plants, drew conclusions somewhat similar to those of Schechner, as did Overton (*14*) working with *Cyperus papyrus*. Pringsheim (*16*), working with succulents, stated that for equal weights old leaves of *Sedum spectabile* lost more water than young leaves, whereas with *Sempervivum braunii* the relation was reversed. Giddings (*8*) found that both the higher and lower leaves of *Silphium laciniatum* transpired less rapidly than leaves borne near the middle of the plant. Koketsu (*11*), using standardized hygrometric paper, found the stomatal transpiring power of Coleus leaves to be greatest at the height of their development. Palladin (*15, p. 139*) has generalized by saying:

[4] Cited by Burgerstein (*1*).

During the period of greatest activity of the leaf, while it is still growing, the rate of transpiration is highest. The reason for this is that the epidermis of young leaves is very permeable to water; transpiration decreases later, but a second maximum is reached when the stomata begin to function. Thereafter the rate of transpiration gradually decreases as the epidermis hardens, in spite of the influence of the stomata.

Pringsheim (*16*) reported the sap concentrations of the old leaves of the two succulents mentioned to be lower than those of the young leaves. Dixon and Atkins (*3*), working with lilacs and other plants, and Chandler (*2*), working with apples and peaches, found the older leaves to have higher sap concentrations. Reed (*17*), however, could find no real difference in the sap concentrations of leaves at opposite ends of new apricot shoots. Hurd-Karrer (*10*) stated that the specific gravity of the sap expressed from the internodes of cornstalks was higher toward the top of the plant. Fernald (*6*) notices a tendency, with irregularities, for the sap of leaves of Philadelphus and privet to increase in concentration toward the tips of branches. In two cases a marked drop in the sap concentrations of the uppermost leaves was shown.

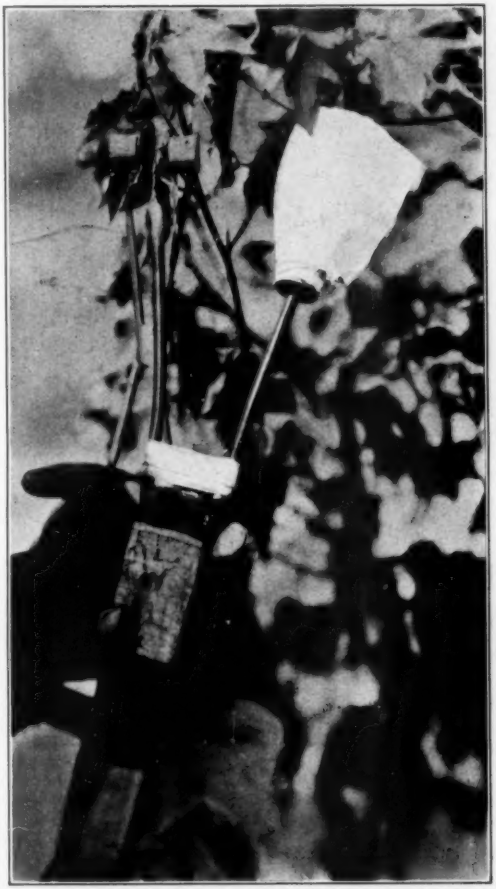

FIGURE 2.—Thermocouple mounting and method of clasping a leaf preparatory to a leaf-temperature reading. The air junction is protected from the direct sunlight by the paper umbrella

METHODS

The temperatures of the leaves were measured in terms of their departures from the temperature of the air. A wall galvanometer, mounted on a screened porch some 40 feet from the experimental plants, was connected by flexible lighting cord to the thermocouples. These were inclosed, except for the junctions, in a piece of semiflexible composition tubing conveniently attached to a wooden

handle. (Fig. 2.) With one of the thermocouple junctions protected from the direct rays of the sun by slwo tightly separated slips of white paper, the second junction was brought firmly into contact with two portions of an upper leaf surface by folding the leaf upward with a pair of cork-tipped crucible tongs. As each leaf was thus secured the galvanometer deflection was recorded by an observer seated on the porch. A more detailed description of the method as well as a discussion of this and other methods of measuring leaf temperatures is given in the paper cited (*5*).

FIGURE 3.—Pima cotton plants grown without branches or bolls. The tall plants were used for the measurements reported in this paper. The leaves of the short plants, which had lost their terminal buds through worm injury, became greatly enlarged and thickened and were very brittle. The sap of the three upper leaves of the short plant had a freezing-point depression approximately 15 per cent greater than that of the leaves of corresponding age from the tall plants. Sacaton, Ariz., September 2, 1927

Four temperature measurements were made on each of the leaves of two plants. (Fig. 3.) Beginning with the lowest leaf that did not show marked signs of deterioration, the temperature of each leaf was measured in turn up the main stalk without regard to the exposure of the leaves to the sun. As soon as the temperatures of all the leaves on one of the plants had been secured the pot was given a quarter turn to bring a new set of leaves into the more direct light and the measurements were then started on the plant in the second pot. This procedure was repeated four times. Immediately following the completion of the temperature measurements all the leaves were gathered for determination of the cell-sap concentration. Each of the sap samples was made up of six leaves, three from each plant for each age group. The mean temperatures of the leaves in each of these groups were averaged for the comparisons. The above-described procedure

provided 24 readings for each temperature mean and six leaves for each sap sample, except that two leaves from one of the plants and one from the other were missing or mutilated. The temperature of the uppermost leaf on one of the plants and the two uppermost leaves on the other (Nos. 45 and 46 in Table 1) were also measured, but these leaves were so small (3 and 4 cm. long) that it was not feasible to collect them for the leaf-sap measurements. Leaves 42 to 44 did not yield sufficient sap for a conductivity determination.

TABLE 1.—*Ages, temperature differences, and freezing-point depressions and conductivities of the expressed sap of leaves at successively higher nodes on the main stalks of two Pima cotton plants grown without branches, at Sacaton, Ariz., September 2, 1927*

Leaves Nos.	Mean age of 6 leaves	Difference between leaf and air temperatures	Freezing-point depression (Δ.) of sap	Specific electrical conductance (κ) at 30° C.
	Days	*° C.*	*° C.*	*Reciprocal ohms*
12-13-14	82	−2.3±0.23	1.52	0.0316
15-16-17	74	−2.7± .27	1.57	.0329
18-19-20	68	−2.5± .20	1.56	.0333
21-22-23	62	−2.8± .30	1.55	.0337
24-25-26	54	−3.1± .24	1.47	.0325
27-28-29	46	−3.1± .20	1.44	.0319
30-31-32	39	−3.5± .16	1.45	.0312
33-34-35	32	−4.0± .16	1.39	.0295
36-37-38	26	−4.1± .11	1.39	.0295
39-40-41	20	−4.1± .15	1.33	.0288
42-43-44	10	−3.7± .13	1.10	
45-46	3	−2.5± .23		

As the concentrations of the cell sap were measured by the methods used extensively by J. A. Harris and his collaborators (e. g., Gortner and Harris, 7), it is not necessary to describe them in detail. At the time of collection the leaves were pressed into tubes and then frozen in a salt-ice mixture for 24 hours. The sap was then expressed from the leaves and the freezing-point depressions and conductivities were determined as soon as possible, the various samples being kept cold until ready for use.

EXPERIMENTAL DATA

The leaf-temperature measurements were made and the leaves were collected for the expressed-sap determinations on September 2, 1927. The temperature measurements were started at 2.17 and ended at 2.47 p. m. A light breeze was blowing while the measurements were being made and the sky was apparently clear, although the sunshine recorder showed a slight but irregular haze insufficient to lower materially the insolation. The air temperature at 2.15 p. m. was 40° C. and the wet bulb was 21°, the relative humidity therefore being 17 per cent. The results of the measurements are presented in Table 1 and shown graphically in Figure 4.

The leaves are grouped in Table 1 according to both age and position on the plant. The ages designated are the means of the leaves in each group. The two plants developed new leaves at very nearly

the same rate, and the corresponding groups of three leaves on the two plants rarely differed in mean age by more than two days. One of the plants was 192 and the other 188 cm. tall on the date of the measurements.

LEAF TEMPERATURE

The leaves (fig. 4) were found to be progressively cooler from the older upward toward the younger until leaves but 10 days old were reached. The oldest leaves had the highest temperatures, 2.3° C. below air temperature. Leaves 26 and 20 days old were the coolest, 4.1° below air temperature. Leaves 10 days and 3 days old were

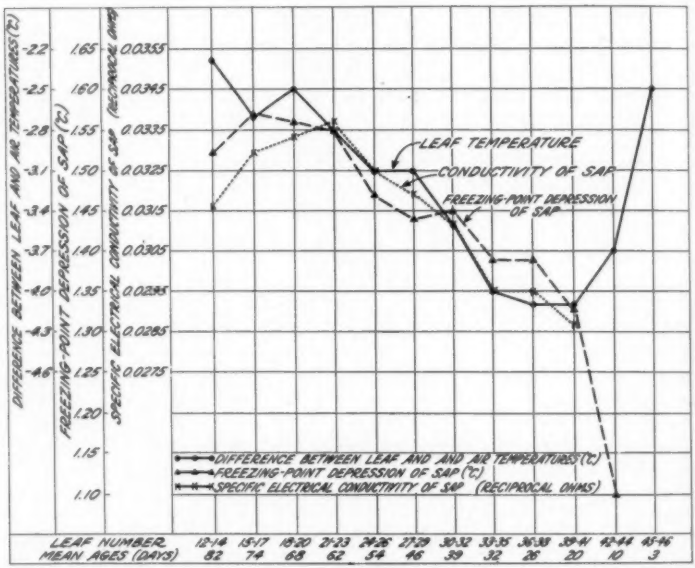

FIGURE 4.—Ages, temperature measurements, and freezing-point depressions and conductivities of the expressed sap of leaves at successively higher nodes on the main stalks of two Pima cotton plants grown without branches, at Sacaton, Ariz., September 2, 1927

warmer than the 20-day-old leaves and had temperatures of 3.7° and 2.5°, respectively, below that of the air.

The probable errors of the means (Table 1) indicate that between successive age groups the temperature differences in general lack statistical significance. It is to be observed, nevertheless, that the temperature curve is both definite in its course and relatively smooth. The variability of the temperatures within each group is to be attributed to the fact that in each group during each of the four series of measurements there were always a number of shaded leaves. Since in each age group there was approximately the same number of shaded or partially exposed leaves, the means doubtless possess a greater significance than that indicated by the probable errors.

OSMOTIC CONCENTRATION

The freezing-point depressions of the expressed sap of the leaves also yielded a curve which is quite definite in character. The osmotic concentrations decrease with the age of the leaves and the height of their insertion. The marked decline in sap concentrations between leaves 20 and 10 days old is noteworthy and suggests that the concentrations in the next younger group, 3-day-old leaves, may have been very low. Between leaves 20 and 10 days old there is a difference of 2.8 atmospheres in the osmotic concentration of the sap.

SPECIFIC ELECTRICAL CONDUCTIVITY

The sap of the leaves of the 62-day group had the highest conductivities, the values for both the older and the younger leaves being lower. The freezing-point depressions indicate a depletion of total solutes in the oldest group of leaves only, whereas the conductivity measurements indicate a depletion of electrolytes in the three oldest groups.

Between the leaves 62 and 20 days old the freezing-point depressions decreased by 14.2 per cent and the conductivities decreased 14.5 per cent. This proportional agreement in change suggested either that the electrolytes and nonelectrolytes decreased in the same proportion or that the nonelectrolytes were present in quantities too small to contribute materially to the freezing-point depressions.

For the purpose of determining which of the possibilities just mentioned was the more plausible, the following computations were made: If it is assumed that the conductivity of these samples was uninfluenced by the presence of organic solutes and colloids it is possible to compute roughly the relative proportions of electrolytes and nonelectrolytes in the samples. By applying the factor 30[5] to each of the conductivity values, results are obtained which represent what theoretically should be the approximate freezing points for the electrolytes alone. Subtracting the products so obtained from the corresponding observed freezing points, the remainders should equal the freezing points of the nonelectrolytes. The latter values for these samples range from 0.47° to 0.58° C. These values, however, are badly out of line with the knowledge of the sugar content of cotton leaves. The amount of sugar typically found in cotton leaves, if all dissolved in the plant sap, would in itself yield a freezing point in the order of but 0.05° to 0.15° C. It must therefore be concluded that conductivity values obtained from plant saps are not closely indicative of the abundance of electrolytes.

Mason (*13*) found that the addition of known quantities of potassium chloride to plant sap did not result in the expected increase in conductance. This he attributed to the viscosity of the sap and attempted to make corrections on that basis. The influence of an organic solute on the conductivity of potassium chloride solutions is illustrated in Table 2.

[5] The factor 30 assumes that sulphates and chlorides were present in the relative proportions found by Harris, Hoffman, and Lawrence (*9*) in Pima cotton leaves at Sacaton and that the predominating cation was sodium.

TABLE 2.—*Specific electrical conductance of 0.1 N potassium-chloride solutions containing increasing quantities of sucrose*

Sucrose concentration per liter of solution (gm.)	Percentage of volume occupied by sucrose	Specific electrical conductance (κ) at 20° C.	Ratio of conductance of solution with sucrose to conductance of solution without sucrose
0	0	0.0117	1.00
79	5	.0103	.88
158	10	.0085	.73
237	15	.0070	.60

Table 2 shows the conductance of a solution containing 0.7 mole of sucrose and 0.1 mole of potassium chloride to be 40 per cent less than that of a pure solution containing the same amount of potassium chloride per liter of solution. For each 1 per cent of the volume occupied by the sucrose the specific electrical conductance was decreased by about 2.5 per cent.

While in no way detracting from the biological significance to be attached to the comparative osmotic concentration of leaf saps, the conclusion is inevitable that neither freezing-point depression nor conductivity values either singly or together furnish reliable information as to the proportion of a freezing point attributable to organic solutes, nor do conductivity measurements on plant sap afford more than an indication with respect to the quantity of electrolytes present.

CORRELATION BETWEEN LEAF TEMPERATURE AND CELL-SAP CONCENTRATION

Values for the leaf-temperature departures and for the freezing points and conductivities of the expressed sap were each obtained for the leaves in age groups from 82 to 20 days. The leaf temperatures and freezing-point values for these groups are found to be correlated to the extent of -0.92 ± 0.114. The coefficient of correlation between leaf temperature and conductivity is -0.86 ± 0.061. Both correlation coefficients are high and indicate a close inverse relationship between the changes in leaf temperature and in the concentration of the expressed sap. In view of the previously cited high correlation between leaf-temperature and transpiration differences, the inference appears justified that there is a close inverse relationship between the changes in sap concentration and the rate of transpiration, i. e., low sap concentrations are conducive to high transpiration rates and reduced leaf temperatures. An agreement even closer is evident in the age groups from 62 to 20 days. This latter series of leaves was looked upon as being neither senescent with regard to cell deterioration nor immature with respect to epidermal characteristics and the development of stomata and intercellular spaces.

While it is possible that the high coefficient of correlation found between leaf temperature and sap concentration may not indicate a causal relationship, there is other evidence which suggests that the rate of transpiration may be intimately associated with the concentration of sap. In previous work (*4*) with Australian salt-

bush and wheat, for example, it was found that when these plants were grown on soils with added sodium chloride there was an increase in the sap concentration with each increment in the salt content of the soil. The sap concentration of the saltbush was increased 42 per cent by this method, and this increase was accompanied by a 44 per cent decrease in the water requirement. Wheat grown on the same soils showed an increase of 72 per cent in the sap concentration and a decrease of 69 per cent in the water requirement between soils with 0.05 and 0.25 per cent of added sodium chloride, but it had a lower water requirement on the control soil than on the 0.05. It has also been found (*5*) that cotton watered with a very saline water had a higher sap concentration and a lower water requirement than when water containing approximately one-fourth as much salt was used. Cotton grown on soils with added sodium chloride has not shown consistent results.

FIGURE 5.—Upper portions of six Pima cotton plants grown without branches or bolls. The two center plants were defoliated on September 2, 1927. The leaves produced after defoliation were lighter green, of finer texture, and much larger than the leaves formed on the control plants during the same period. Sacaton, Ariz., September 25, 1927

LEAF DEVELOPMENT FOLLOWING DEFOLIATION

It has been noted, particularly perhaps in arid regions, that after trees or other plants have been heavily pruned the new leaves that develop are frequently larger and of a more mesophytic character than those cut away. While such reactions may be looked upon as an indication that pruning in some manner exerts an invigorating effect upon the plant, the measurements just reported suggest an alternative explanation on the basis of water relations. As may be observed in Figure 5, the new leaves produced by the cotton plants after the leaves had been removed for sap determinations were quite different from leaves produced during the same period by otherwise similarly treated plants from which the leaves had not been removed.

The upper portions of the two Pima cotton plants are shown as they appeared 23 days after defoliation. The new leaves which appeared on the defoliated plants were of a softer texture, very

much larger, thinner, and lighter green than those on the control plants shown at either side. The comparative elongation rates and the rate of new leaf development are given in Table 3.

TABLE 3.—*Comparison between 2 defoliated and 15 control Pima cotton plants, with respect to elongation rates and the unfolding of new leaves, before and after September 2, when defoliation took place*

MEAN DAILY ELONGATION OF PLANTS (CM.)

Description of plants	Before defoliation				After defoliation				
	Aug. 8–14	Aug. 15–21	Aug. 22–28	Aug. 29–Sept. 1	Sept. 2–4	Sept. 5–11	Sept. 12–18	Sept. 19–25	Sept. 26–Oct. 9
Control	2.4	1.6	1.7	1.3	1.6	1.6	1.3	1.4	0.6
Defoliated	2.6	1.5	1.5	1.3	1.0	1.0	1.6	1.5	.6

MEAN DAILY NEW LEAF FORMATION PER PLANT (NUMBER)

Control	0.46	0.31	0.23	0.38	0.27	0.46	0.34	0.23	0.10
Defoliated	.43	.29	.29	.38	.50	.57	.43	.29	.11

The height of plant measurements show that the elongation rates of the defoliated plants were less than those of the control plants for approximately 10 days immediately following defoliation, after which period it appears that the defoliated plants may have grown slightly faster than the controls. Defoliation did not retard, even temporarily, the formation of new leaves. Between August 8 and September 2, before defoliation, there were unfolded on both the experimental and the control plants a mean of 8.5 new leaves per plant, whereas between September 2 and October 10, after defoliation, the control plants produced 9.5 leaves per plant and the defoliated plants produced 12 leaves each. Comparative measurements of the areas of the leaves on the control and defoliated plants were not secured, but it was estimated that the new leaves on the defoliated plants attained an area not less than three times that of the leaves of the same age on the control plants.

The availability of considerable quantities of reserve translocatable materials may be looked upon as prerequisite to an increased leaf expansion as marked as that shown by these plants. The complete defoliation of plants without such reserves would doubtless have been followed, at least temporarily, by weak growth if not by death. Ludwig (*12*), who studied the relation of defoliation to yields of cotton, reported that in irrigated plots many plants died as a result of late-season defoliation. There is some evidence, aside from the reaction itself, which suggests that the writer's group of plants were operating with an abundance of hydrolyzable material in reserve, the presence of which can be most readily attributed to the fact that they were grown without bolls. It is improbable, however, that considerations involving nutritional relationships would be of great value in explaining the reactions of the plants to defoliation, since plants most abundantly supplied with leaves should, with other things equal, be most able to extend their vegetative development

Figure 5 furnishes convincing evidence that the lower leaves of the control plants exerted a marked influence upon the development of the leaves above. It is not difficult to conceive that the old leaves, approaching senescence, may have liberated growth-inhibiting substances which were translocated upward to the younger leaves to depress their growth and prevent them from attaining the luxuriant development which is so marked in the case of the leaves formed after defoliation on the experimental plants. A hormone theory in this case, however, would require an assumption as to both the presence and the method of action of an unknown chemical substance and therefore must be of uncertain applicability.

The freezing-point depression determinations showed that the sap in the upper leaves of these plants was less concentrated than that in the lower leaves. This difference in osmotic concentration would place upper leaves at a disadvantage with respect to older leaves in their ability to withdraw water from the transpiration stream when old leaves were present and not fully turgid. Chandler's tomato experiment (*2*) shows this to be more than a theoretical consideration. In his experiment he placed the roots of one of a pair of approach-grafted plants in a sugar solution to increase its sap concentration and the roots of the other in distilled water. When the roots of both were exposed to the air the plant from the sugar solution was able to draw water through the graft and remain turgid at the expense of the companion plant, which wilted. In addition to the difference in osmotic concentration, the upper cotton leaves were also at a disadvantage in their water relations as a result of their greater height of insertion, which would impose both a difference in the lift and additional frictional resistance to water movement.

Not only were the upper leaves of the control plants at a disadvantage with respect to their ability to obtain water, but they were also less able to retain it against transpiration. The leaf-temperature measurements showed higher transpiration rates for the younger than for the older leaves. On those days in which the transpiration rates of these plants exceeded the rates of water absorption and conduction the upper leaves always showed the first signs of wilting.

The difference in the water relations of the young and old leaves appears sufficient to account for the difference in the growth characters of the leaves on the two sets of plants as well as of the upper and lower leaves of the control plants. Leaves of plants under mesophytic conditions are almost always larger, thinner, and of finer texture than the leaves of plants in exposed or dry habitats, as is amply illustrated in the literature. The two factors limiting the quantity of water which may be supplied to transpiring leaf surfaces are the rate of water absorption and the rate of its conduction to the leaves. During periods of high transpiration either one or both of these rates may become limiting factors in the amount of water maintained in leaf tissues. When such shortages exist it is natural that a competition for water between leaves should result. The evidence obtained from the cell-sap concentration determinations indicate that older cotton leaves, because of their relatively high sap concentration, were osmotically at an advantage over younger leaves in their ability to withdraw water from the transpiration stream, and also, as indicated by the temperature measurements, the older leaves required less water to meet the demands of transpiration.

The evidence furnishes confirmation of the view that xerophytic leaf characters under conditions of water stringency, internal or external, should be looked upon as being consequences of the environment rather than as fortuitous adaptations which peculiarly fit plants to their environment.

SUMMARY

The sap concentrations and the temperatures of Pima cotton leaves varying in average age from 82 to 3 days were measured. These leaves were at successively higher nodes of the main stalks of plants grown without branches or bolls.

The freezing-point depressions of the expressed sap from the leaves decreased from 1.57° C. for leaves 74 days old to 1.10° for leaves 10 days old.

The specific electrical conductivities of the same saps increased from 0.0316 reciprocal ohms for leaves 82 days old to 0.0337 for leaves 62 days old, and then decreased to 0.0288 for leaves 20 days old.

Young leaves were found to be cooler than old ones. The temperature of leaves 82 days old was 2.3° C. below that of the air, whereas the temperature of leaves 26 and 20 days old was 4.1° below. The temperature of very young leaves, 3 days old, was approximately the same as that of leaves 82 days old. The coefficient of correlation between the temperature and the sap concentration of leaves from 82 to 20 days old was -0.92 ± 0.035 for the freezing point and -0.86 ± 0.061 for conductivity. Reference is made to an earlier paper (*5*), in which it was shown that the differences in the temperatures of cotton leaves with different transpiration rates were inversely proportional to the transpiration differences.

The plants defoliated for the leaf-sap measurements subsequently developed leaves of a more mesophytic character and of approximately three times greater size than the coincident leaves on similarly treated plants that had not been defoliated. The leaf-temperature and sap-concentration measurements are looked upon as furnishing an explanation of the stimulated leaf development on the basis of differences in the water relations of the new leaves on the two sets of plants. Young leaves of the cotton plants with their low sap concentration were less able than the old leaves to obtain water from the transpiration stream, and their higher rate of transpiration served to increase their requirements per unit of area.

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DISEASES OF ROSE CAUSED BY SPECIES OF CONIOTHYRIUM IN THE UNITED STATES [1]

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INTRODUCTION

In the examination and study of many types of cankers on diseased rose stems the attention of the writer has been attracted to the great prevalence of species of Coniothyrium as the cause of such cankers. Because of the many points of similarity among the three diseases popularly known as stem canker, graft canker, and brand canker, attributed respectively to *C. fuckelii* Sacc., *C. rosarum* Cke. and Hark., and *C. wernsdorffiae* Laub., the question frequently arises as to the identity of the particular organism causing a certain type of canker. The present study was undertaken to determine by a comparison of the symptoms of disease and of the morphological and physiological characters of the causal fungi what distinguishing features might be of value in diagnosing the diseases. The immediate occasion for the study was the recent discovery, in the United States, of brand canker, caused by *C. wernsdorffiae*, hitherto reported in Europe and Canada.

SURVEY OF LITERATURE

Brand canker was originally described in 1905 by Laubert (*10*) [2] as a disease attacking branches of Rosa in many localities in Germany. From a morphological study of the organism causing the canker, he concluded that the fungus was a species of Coniothyrium. The species, however, did not correspond with *C. fuckelii*, which had been reported by Saccardo (*19*) as occurring on dead or wilted branches of Rosa and other hosts. Therefore Laubert established the fungus as a new species, *C. wernsdorffiae*. Kock (*9*) in the same year reported a disease on tea roses in Austria which he ascribed to *C. fuckelii*. He believed that this was the same disease as that found by Laubert and that the slight difference between the spore measurements given for Saccardo's fungus and those of his own Austrian specimens was an insufficient basis for establishing a new species. He would make the fungus causing the disease merely a variety of *C. fuckelii*. His spore measurements and description of the disease symptoms corresponded rather closely with those given for *C. wernsdorffiae* by Laubert (*10*), and it would seem that both writers were describing the same disease.

In a later publication Laubert (*11*) compared the fungus causing the brand canker with *Coniothyrium fuckelii*. He considered the latter fungus a harmless saprophyte with pycnidia and spores much smaller than those of *C. wernsdorffiae*.

Güssow (*4, p. 226*) described the symptoms and causal organism of a similar disease found upon hybrid tea roses and Wichuraianas in

[1] Received for publication Dec. 30, 1929; issued May, 1930. These studies were conducted in cooperation with the Department of Botany, Brown University, Providence, R. I.

[2] Reference is made by number (italic) to "Literature cited," p. 826.

Ireland. He considered the causal organism to be *Coniothyrium fuckelii*, since his spore measurements "practically agreed with the size of the spores of Saccardo's fungus." Moreover, he believed that the disease on the Irish specimens was identical with that observed by Laubert and agreed with Kock that Laubert was not justified in establishing a new species. From Güssow's description and the accompanying illustrations it would seem very probable that his specimens were infected by *C. fuckelii*, as he stated, and that he was not dealing with the disease described by Laubert.

In Rostrup's Danish Fungi (*13, p. 436*) appeared the following note concerning *Coniothyrium wernsdorffiae:* "A true parasite, attacking the bark of the branches of cultivated *Rosa* spp. for the first time found 11/6/03 [June 11, 1903], quite common."

O'Gara (*16*) found a species of Coniothyrium causing cankers on rose in the United States which proved by cultural methods and cross inoculations to be identical with a species occurring on cankers on apple twigs. The fungus appeared to correspond in every respect with the description of *C. fuckelii* given by Saccardo. O'Gara stated that the organism fruited readily in cultures, "producing typical pycnidia and spores varying somewhat in size, depending upon the medium, but all within the limits of the species."

From these investigations it seemed apparent that two different species of Coniothyrium caused diseases of rose. Since the time of O'Gara's investigations, however, several writers have stated that *C. wernsdorffiae* was probably identical with *C. fuckelii*.

Saccardo (*19*) designated *Coniothyrium fuckelii* as the "spermagonial" form of *Leptosphaeria coniothyrium* (Fckl.) Sacc. The same statement was made by Massee (*15*). Experimental inoculations and cultures to determine the exact relation of the perithecial and pycnidial forms were successfully made by Stewart (*20*) on various species of Rubus. Martin (*14*), in a discussion of the polymorphism of *L. coniothyrium* on rose, reported Coniothyrium as one of the phases of the fungus. So far as known at the present time no perfect stage for *C. wernsdorffiae* has been reported.

In 1925 the first reports of the occurrence of brand canker in North America were published by Howitt (*6*) and by Martin,[3] following the identification of *Coniothyrium wernsdorffiae* as the cause of cankers on material collected in Guelph, Ontario. Later the occurrence of the disease in the United States was reported by Jenkins and Martin (*8*)[4] and was tentatively described by Jenkins (*7*), Westcott,[5] and the writer (*23*). Its occurrence in the Netherlands (*18*) was also reported.

The third disease of rose attributed to a species of Coniothyrium was described by Vogel (*22*) as graft disease. From his study of the causal fungus and from the results of his inoculation experiments he concluded that the disease was caused by *C. rosarum*. This fungus was originally reported by Cooke and Harkness (*2*) as occurring on stems of Rosa in California. No report of the perfect stage of this fungus has been made.

[3] MARTIN, G. H. DISEASES OF FOREST AND SHADE TREES, ORNAMENTAL AND MISCELLANEOUS SHRUBS IN THE UNITED STATES IN 1924. U. S. Dept. Agr., Bur. Plant Indus., Plant Disease Rptr. Sup. 42: 360. 1925. [Mimeographed.]

[4] JENKINS, A. E., and MARTIN, G. H. BRAND CANKER OF THE ROSE APPEARS IN THE UNITED STATES. U. S. Dept. Agr., Bur. Plant Indus., Plant Disease Rptr. 10: 24–25. 1926. [Mimeographed.]

[5] WESTCOTT, C. AN EPIPHYTOTIC OF BRAND CANKER OF ROSES CAUSED BY CONIOTHYRIUM WERNSDORFFIAE LAUBERT. U. S. Dept. Agr., Bur. Plant Indus., Plant Disease Rptr. 10: 38–40. 1926. [Mimeographed.

DISTRIBUTION OF THE DISEASES

In the summer of 1926 a serious epiphytotic of a disease similar to that originally described in Germany by Laubert as brand canker caused by *Coniothyrium wernsdorffiae* was discovered by the writer on climbing roses in the floriculture gardens at Cornell University, Ithaca, N. Y. (Fig. 1.) No report of the occurrence of this disease in the United States had been published at that time. Later the disease was reported by Jenkins and Martin (*8*) as occurring also in Minnesota and Pennsylvania. So far as known at the present time, this disease occurs principally on out-of-door roses. Jenkins and Martin (*8*) reported it on rose plants grown in the horticulture greenhouses of the Minnesota Agricultural College. With this exception all reports and collections have been from garden plants.

FIGURE 1.—Brand canker, showing fruiting bodies of the causal fungus

The disease commonly known as stem canker (fig. 2) and caused by *Coniothyrium fuckelii* is reported as widespread throughout the United States. It is found at some time in nearly every rose garden and in many greenhouses. It is more prevalent, however, on hybrid tea roses than on other types of roses.

The so-called graft disease attributed by Vogel to *Coniothyrium rosarum* was considered to be limited to rose grafts in forcing frames and greenhouses and to plants in the rose garden which had contracted the disease while in the greenhouse but which proved to be partially resistant. During 1927 a similar disease (fig. 3) was particularly prevalent in greenhouses throughout the middle and eastern parts of the United States.

The accompanying map (fig. 4) shows the distribution of these three diseases in the United States as known at the present time. This map was prepared from data published by Martin and Jenkins [6] and from the results of the writer's studies. Undoubtedly the diseases are more widely distributed than this map signifies, but possibly they are not sufficiently serious to attract particular attention in those States from which a report of their occurrence has not been received.

[6] MARTIN, G. H., and JENKINS, A. E. PRELIMINARY LIST OF FUNGI AND DISEASES OF ROSES IN THE UNITED STATES. U. S. Dept. Agr., Bur. Plant Indus., Plant Disease Rptr. 63: 357. 1928. [Mimeographed.]

MATERIAL USED IN THE PRESENT STUDY

The material used in the present study was collected from various localities in the United States and from plants grown under widely differing conditions of environment. The specimens of brand canker

FIGURE 2.—Stem canker which started from an infection of the wound at the lower end of the prickle

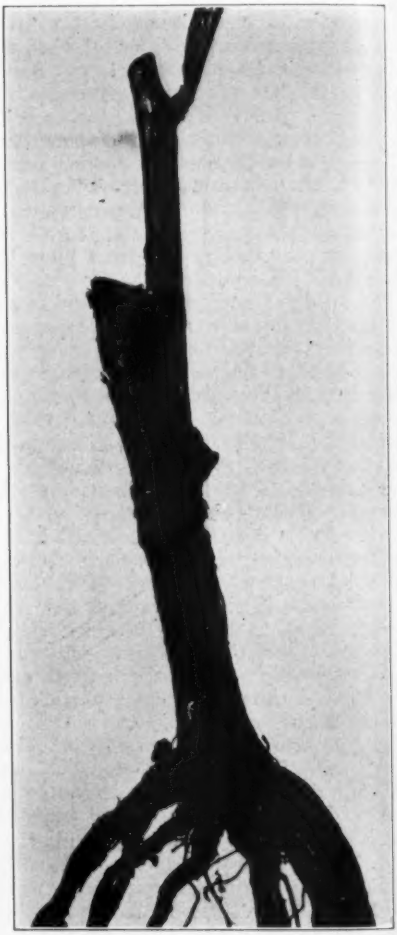

FIGURE 3.—A young grafted rose plant bearing graft canker along the side of the scion nearest the stock

were collected by the writer at Ithaca, N. Y., and were a portion of the material reported by Jenkins and Martin (*8*), who attributed the disease to *Coniothyrium wernsdorffiae*. The term "stem canker" is

applied throughout this study to the disease which is commonly considered to be caused by *C. fuckelii*, the imperfect form of *Leptosphaeria coniothyrium* Sacc. A few specimens of *L. coniothyrium* were studied by the writer and were compared with exsiccati specimens among the "Fungi Saxonici" collected by K. W. Krieger. In the study of this material, cultures from the ascospores produced a species of Coniothyrium which corresponded with that isolated from the stem cankers. Therefore the fungus causing such cankers has been called *C. fuckelii*. The name "graft canker" has been applied only to the disease characterized by the formation of cankers at the union of stock and scion. The fungus isolated from such cankers was compared with that occurring on specimens from the herbarium of the Iowa State College of Agriculture and Mechanic Arts,[7] which were collected by I. H. Vogel, who attributed the disease to *C. rosarum*.

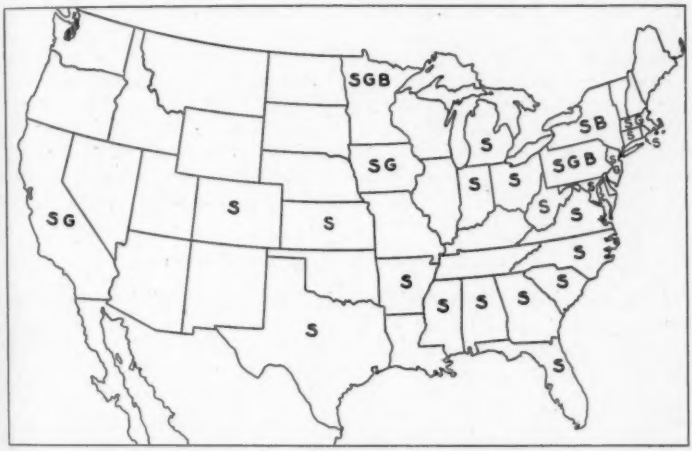

FIGURE 4.—Map showing distribution of stem canker, graft canker, and brand canker diseases in the United States. S=stem canker; G=graft canker; B=brand canker

BRAND CANKER

SYMPTOMS

The symptoms of the disease known as brand canker have been described in detail by various investigators (*9*, *10*, *11*, *23*). Therefore it is necessary in this article only to call attention to those characters which are distinguishing features.

The cankers can be readily detected, even by a casual observer. This is due to the fact that the light or warm buff [8] of the central portion and the taupe-brown or dull purplish black margin stand out in sharp contrast with the green of the adjacent healthy stem.

Small longitudinal slits in the bark of the diseased area, caused by the protruding of the pycnidial ostioles, are characteristic of these cankers. Only in rare cases were the spores of the fungus extruded

[7] The writer acknowledges the courtesy of Dr. I. E. Melhus and Dr. J. C. Gilman, of the Iowa State College of Agriculture and Mechanic Arts, for the loan of this material.

[8] The color terms mentioned in the text are according to the following publication: RIDGWAY, R. COLOR STANDARDS AND COLOR NOMENCLATURE. 43 p., illus. Washington, D. C. 1912.

upon the bark in sooty masses. In Laubert's description of the disease (*10*), however, he stated that the spores are frequently collected on the surface of the bark "in Form sehr kleiner russschwarzer Fleckchen." Since the majority of the cankers of this disease which were examined by the writer were of comparatively recent infection, it may be that the accumulation of the spores occurs only on older cankers. Laubert also mentions the occurrence of wound callus at the margin of 2-year-old cankers (*10*). No such callus formation has been found in connection with the cankers examined by the writer. This again may be due to the fact that young cankers only have been observed in this country.

MORPHOLOGY OF THE CAUSAL FUNGUS

Mycelium

In the host tissue the mycelium of the causal fungus showed no distinguishing features. In culture, however, although the mycelium varied with the kind of agar used, certain distinguishing features of color and amount of growth were apparent. The aerial mycelium of the fungus grew exceedingly slowly on all the media used but eventually became luxuriant. On corn-meal and potato agars it was light gray; on malt, dextrose, and synthetic [9] agars, a greenish gray turning to brownish gray. On sterilized sliced potato, the actively growing mycelium was a very light gray, becoming darker with age until, in an old culture, it was almost black.

Formation of Pycnidia and Spores

The formation of a pycnidium is first indicated by close interweaving and anastomosing of numerous rapidly growing hyphae. This usually occurs in some intercellular space at a point within the zone of the three outermost cell layers below the epidermis of the diseased area. As this tangled mass or primordium grows larger, the epidermis together with the two or three underlying cell layers becomes widely separated from the inner layers of the bark tissue. In the center of the primordium a cavity is formed by the absorption of some of the hyphal cells. By this lysigenous activity the cavity is gradually increased in area until it attains its characteristic size. In a mature pycnidium the cells lining the cavity are hyaline, thin walled, and finely granular. These cells are sporogenous in function. There is no pigmentation in the cell walls of this tissue. The next two or three layers are similar to these in appearance. These few layers of sporogenous cells are closely packed and more or less regular in shape. This arrangement, together with the granular contents of the cells, gives the appearance, in sections, of a subhyaline ring of tissue surrounding the cavity. This ring is especially characteristic of the pycnidia formed by the brand-canker fungus. (Fig. 5.) Adjacent to these layers are several rows of larger cells, devoid of granular cell contents and containing a brown pigmentation in the cell walls. These layers of cells form the enveloping wall of the pycnidium.

The pycnidial cavity may vary considerably in shape, and the pycnidia are usually so closely grouped that they appear stromatic.

[9] The synthetic agar was made according to the formula given by Leonian (*12*) as follows: Dihydrogen potassium phosphate, 1.25 gm.; magnesium sulphate, 0.625 gm.; peptone, 0.625 gm.; maltose, 6.25 gm.; malt extract, 6.25 gm.; distilled water, 1,000 c. c.; agar, 1.5 to 2 per cent.

(Fig. 6.) The fungus is characterized by complex pluriloculate pycnidia rising from several confluent primordia. Mature pycnidia are frequently found in which the intervening walls between the aggregate pycnidia have broken down, leaving one large irregular cavity.

In Laubert's description of *Coniothyrium wernsdorffiae* (*10, p. 460*) he described the pycnidia as "mit ektostromaartiger Papille," penetrated by a broad ostiolate canal. From a study of sections from a number of pycnidia on the host tissue it was found that, when the formation of the pycnidial cavity is completed, there develops at the top of the pycnidium, just below the epidermis, a small cone-shaped mass of thin-walled cells forming a "buffer tissue" (*3*). (Fig. 5.) The walls of these cells are at first hyaline, but as the tissue grows and finally ruptures the epidermis, the walls become subhyaline, then brown, and finally almost black. The cells along the central line of

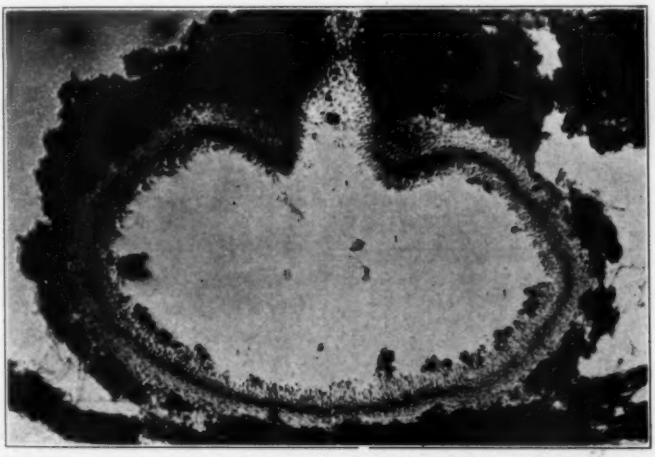

FIGURE 5.—Pycnidium of the brand-canker fungus showing the ostiole developing through the buffer tissue and the apparent ring of tissue formed by the layers of sporogenous cells. × 160

this tissue dissolve or disintegrate, and a canal ending in a papillate ostiole results. From the cells lining this canal are produced short filamentous cells resembling the periphyses commonly found in papillate or beaked ostioles of perithecia. These periphyses may disappear as soon as the pycnidium becomes mature, but the buffer tissue often remains for some time. It is this buffer tissue which caused Laubert to describe the pipilla as "ektostromaartige."

In culture the pycnidia show more distinctly than in nature the beaklike papilla through which the ostiolar canal develops. The buffer tissue, however, was present only preceding the formation of the papilla and ostiole. It was frequently found that a secondary cavity was formed in the beaklike papilla. (Fig. 7.) The two cavities are at first separated from each other by a wall several cell layers in thickness. Eventually, however, the separating wall is broken down and an ostiole is formed through the outer wall of the upper cavity.

Laubert (*10*) further stated that the spores were abstricted from the innermost layer of cells in the pycnidial cavity and that sporophores were lacking. The manner in which the spores are produced in certain species of Coniothyrium has been considered by Von Höhnel

FIGURE 6.—Aggregate pycnidia of the brand-canker fungus. × 160

(*5*) as the basis for separating these species of the genus into a new genus, Sclerothyrium. The distinguishing characters of this new genus are the absence of conidiophores and the endogenous formation of the spores.

In the present study of the brand-canker fungus it has been found that the spores are produced by budding from the innermost layer of cells lining the pycnidial cavity. (Fig. 8, A.) These cells appeared to be coated with a gelatinous substance, probably resulting from the lysigenous activity in the process of cavity formation. The first indication of spore formation is the protrusion of a very slender

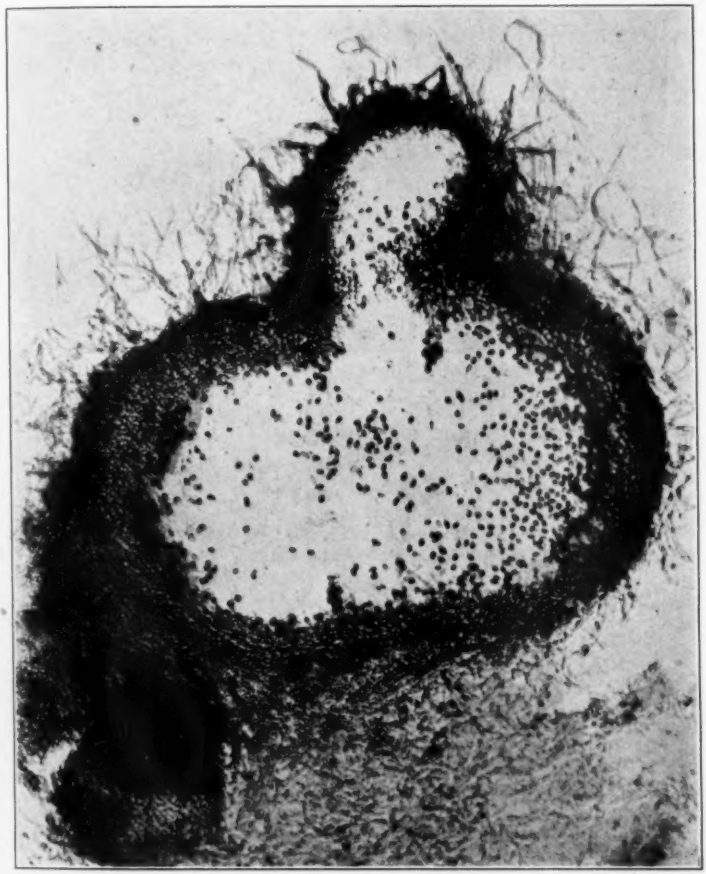

FIGURE 7.—A pycnidium of the brand-canker fungus produced in culture. A secondary cavity has formed in the beaklike papilla. × 160

filament of cytoplasm from a cell of the sporogenous layer into the gelatinous coating. The tip of this filament gradually swells and finally emerges from the coating. It then has the ovoid or spherical form of a mature spore and has a thin gelatinous film surrounding it. This ovoid tip remains connected with its parent cell by a thin strand of cytoplasm until the spore reaches maturity. A wall is then formed between the spore and its parent cell and the cytoplasmic strand

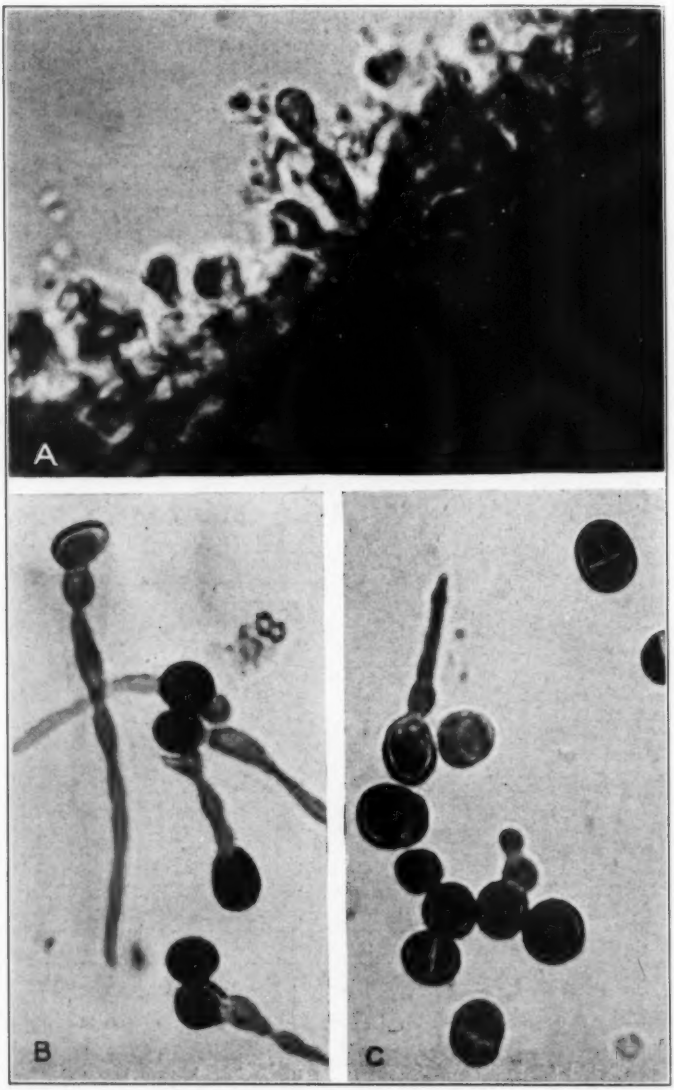

FIGURE 8.—A, Section of the sporogenous layer of cells in a pycnidium of the brand-canker fungus, showing near the center a developing spore. × 2,500. B, Germinating spores of the brand-canker fungus. × 1,300. C, Spores of the brand-canker fungus which became septate before germination. × 1,300

disappears. This type of spore formation has been described for certain species of both Phoma (*1*, *24*) and Coniothyrium (*17*), which were placed respectively in the genera Sclerophoma and Sclerothyrium by Von Höhnel (*5*). It is apparent that the spores are not formed endogenously, nor are they produced on typical conidiophores. It seems advisable, however, to designate the species as of the genus Coniothyrium until a revision of Von Höhnel's Endogenosporae has been made.

The ovoid spore remains hyaline until it reaches its mature size, at which time pigmentation begins to appear. The pigmentation is confined to the exospore of the spore wall. The spore contents remain colorless.

The spores are abstricted singly from the parent cells. Because of the gelatinous film surrounding each spore, they frequently adhere end to end and give the appearance of chains of spores produced from one cell.

Size and Shape of Spores

The question of spore size has been considered of importance in determining whether the three rose diseases are caused by the same or different species of Coniothyrium. Laubert's measurements for *C. wernsdorffiae* (*10*) are given as 4.5μ to 6μ by 5μ to 8μ. Kock (*9*) found that the spores of his specimens gave an average length of 5.6μ and width of 4μ. The length of the spores was very variable, some as long as 5.7μ, others 6.6μ, and still others 4.7μ.

In the present study spores of the brand-canker fungus on the host were obtained by crushing on a slide several mature pycnidia. The spores were mixed thoroughly, and a few loopfuls were mounted on a slide in a solution of potassium acetate, 10 gm.; pure glycerine, 200 c. c.; 95 per cent alcohol, 300 c. c.; distilled water, 500 c. c. The spores were allowed to stand in a drop of the solution on a slide for 24 hours, and 50 spores from each of two slides were then measured with a filar micrometer. The measurements of the two sets were then combined, and the means and standard range for the 100 spores were calculated. One hundred spores from several pycnidia produced in polysporic cultures on potato agar were similarly measured. Extruded spores only were used for this series of measurements. The origin of the material used and the results obtained are shown in Table 1. The mean ratio of length to width is given in Table 2.

Table 1.—*Biometric data on spores of fungi from brand canker, stem canker, and graft canker*

Fungus	Source of spores	Length			Width		
		Mean (μ)	Standard deviation	Standard range (μ)	Mean (μ)	Standard deviation	Standard range (μ)
Brand canker	Host; garden; New York	5.29	0.533	4.76–5.82	3.83	0.36	3.47–4.19
Do	Culture	5.61	.667	4.94–6.28	4.18	.385	3.79–4.57
Stem canker	Host; garden; District of Columbia.	3.09	.358	2.73–3.45	2.15	.26	1.89–2.41
Do	Host; garden; Rhode Island.	2.98	.427	2.55–3.41	2.01	.276	1.73–2.29
Do	Host; grafted plants; greenhouse; Georgia.	3.27	.405	2.87–3.68	2.18	.281	1.89–2.46
Do	Host; cuttings; greenhouse; Maryland.	3.37	.436	2.93–3.81	2.16	.292	1.87–2.45
Do	Culture	2.75	.368	2.38–3.12	2.02	.41	1.61–2.43
Graft canker	Host; greenhouse; Pennsylvania.	3.04	.426	2.61–3.47	2.17	.296	1.87–2.47
Do	Host; greenhouse; Iowa	3.18	.389	2.79–3.57	2.17	.273	1.89–2.44
Do	Culture	3.2	.376	2.82–3.58	2.13	.234	1.89–2.36

TABLE 2.—*Ratio of length to width for spores of fungi from brand canker, stem canker, and graft canker*

Fungus	Source of spores	Mean ratio
Brand canker	Host	1.35
Do	Culture	1.32
Stem canker	Host	1.47
Do	Culture	1.35
Graft canker	Host	1.39
Do	Culture	1.49

The actual range of the spores measured was 4.0μ to 7.2μ by 3.0μ to 5.4μ. There is a slight difference between these measurements and those given by Laubert (*10*) for *Coniothyrium wernsdorffiae*. This is probably due to the fact that spores larger than 7.2μ are exceedingly rare. Many pycnidia from cultures were examined, the pycnidia being crushed on a slide in an attempt to force out all the spores. In one case where two large pycnidia were crushed on a slide, only 12 spores on the entire slide could be found whose length exceeded 7.2μ; these ranged from 7.4μ to 9.4μ. Another slide with two crushed pycnidia showed a larger number of spores exceeding 7.2μ in length. The largest spore measured was 10.6μ by 10.4μ. The width also varied in these larger spores. Their actual measurements may be given as 7.4μ to 10.6μ by 5.2μ to 10.4μ. Among the spores on the slide, however, were many that had begun to germinate. It is therefore possible that the large size of these spores is merely a phenomenon of germination and that these measurements do not indicate the normal size of a mature spore. Some of these large spores were uniseptate. As will be mentioned later, this septation sometimes occurs preceding germination.

PHYSIOLOGY OF THE CAUSAL FUNGUS

DESCRIPTION OF CULTURES ON VARIOUS MEDIA

MALT AGAR.—The fungus produced a moderately abundant, fluffy, aerial mycelium which in mass was greenish gray. Pycnidia were rare and were first noticeable about four weeks after inoculation. Another three or four weeks of growth was necessary to bring them to maturity.

CORN-MEAL AGAR.—A slight amount of grayish mycelium was produced. Only a few pycnidia were formed. The rate of growth was relatively the same as on malt agar.

POTATO AGAR.—The mycelium was moderately abundant, white to mouse gray, and mature pycnidia were present about six weeks after inoculation. This medium proved very satisfactory for the production of pycnidia and spores.

STERILIZED POTATO SLICES.—A dense, fluffy, mouse gray to dark gray aerial mycelium was formed, producing abundant mature pycnidia in six to eight weeks after inoculation. The pycnidia were large with a pronounced tendency to aggregate or become complex. The spores were exuded in the form of spore horns.

DEXTROSE AGAR.—The dextrose agar was made with 2 per cent dextrose and 2 per cent agar. A moderate amount of greenish gray aerial mycelium was produced, but no fruiting bodies were developed to maturity. A few appeared to form in the mycelium along the edge of the agar slant, but they failed to develop spores.

SYNTHETIC AGAR.—The synthetic medium developed by Leonian (*12*) for the production of pycnidia in cultures of Sphaeropsidales was used. The medium was more satisfactory than the others with the exception of the sliced potato. A fluffy, greenish gray mycelium was formed, and pycnidia appeared after about eight weeks.

ROSE-STEM AGAR.—A medium made from a decoction of rose stems with agar was tried, but the results did not prove of value. No mature pycnidia were produced.

STERILIZED ROSE STEMS.—Stems of climbing and hybrid tea roses were cut into convenient lengths, placed in flasks with 10 to 20 c. c. of distilled water, and sterilized. When cool the stems were inoculated with mycelium, with spores, or with entire pycnidia. Growth was very satisfactory, and pycnidia were produced abundantly.

SPORE GERMINATION

Germination tests were made on several different media. In hanging-drop cultures with tap water the spores showed evidence of germination in 24 to 36 hours. (Fig. 8, B.) In distilled water the number of germinating spores was very much smaller and the rate of germination was very much slower. Tests were also made by sowing spores on a thin film of agar on a microscope slide. The agar mounts were kept moist in a Petri dish and were examined at 24-hour intervals. The agars used were malt, potato, dextrose, and Leonian's synthetic. The spores germinated on all these agars with about the same readiness as in tap water.

Germination was preceded by a swelling of the spores. Very frequently this increase in size was followed by septation. (Fig. 8, C.) The first septum usually originated as a slight separation of the cytoplasm near the center of the spore. The separation gradually extended outward to the spore wall, and a definite septum then became apparent. In some cases the spores became constricted at the septum. A second septum sometimes appeared, either parallel with or at right angles to the first septum. In these septate spores germination may take place from each of the cells. The tendency of spores of Coniothyrium species to become septate has been previously reported by Archer (*1*), but no extensive investigations have been conducted to determine the possible relation of septation to changes in environment. It is apparent, however, that spores of certain species of Coniothyrium may show the same tendency toward septation as do those of species of Sphaeropsis.

PATHOGENICITY OF THE ORGANISM

INOCULATION EXPERIMENTS

Since facilities for growing roses for inoculation under greenhouse conditions were not available to the writer, it was necessary to devise other means for growing rose cuttings. Several pots of sterilized sand were prepared, and in these pots were placed cut stems of a climbing rose. The leaves were removed and the cuttings were allowed to grow about one week before they were inoculated. One series of inoculations was made by placing the inoculum, composed of a portion of a mature pycnidium with exuding spores, directly in the axil of a bud without injuring the bud or bark in any way. In a second series the inoculum was introduced into the stem tissue through a wound in the bark made by a sterile scalpel. The inoculum used was obtained from polysporic cultures grown on sterilized potato slices. In each series of experiments one pot with a cutting was set aside as a control. In all cases the cuttings were kept in a moist atmosphere under bell jars.

All the cuttings produced new healthy leaves from the two uppermost buds. With the exception of those buds in the axils of which the inoculum was placed, the buds remained green and healthy, although they developed no leaves. The inoculated buds soon turned brown and in about six weeks were found to be covered with pycnidia. The spread of the fungus in the bark tissue, however, was very slow. The infected bark at first became a reddish brown, then a dark brown. As

the pycnidia appeared and the tissue of the affected areas became dried and dead, the color of the central portion changed to a light brown with a margin of reddish brown. Before the cankers had reached any considerable size, however, the cuttings died. The fungus was reisolated from the infected areas. On the canes that were inoculated through wounds made with a sterile scalpel, the infected areas increased in size more rapidly and pycnidia were produced more abundantly.

At the same time a series of similar inoculations was made on red-raspberry canes. In all cases small cankers appeared on the bark of the canes before the death of the cuttings. As the bark became dried following infection, it peeled off in shreds. This characteristic was noted by Laubert (*10*) in cankers on rose canes caused by *Coniothyrium wernsdorffiae*. The small prickles on the raspberry canes became infected and in some cases a pycnidium was formed within the prickle, the fungous hyphae thus taking the place of the disintegrated host tissue. Cankers were formed from infected buds as well as from inoculated wounds.

No reference in literature could be found to the occurrence of *Coniothyrium wernsdorffiae* on parts of rose plants other than stems or branches. Therefore, tests were made with hybrid tea and climbing rose leaves, as well as with the hips of *Rosa rugosa*, to determine whether the fungus would infect them and possibly overwinter in this way. In no case, however, did the fungus infect the leaves or hips in a moist chamber.

Penetration of the Host

In Laubert's (*10*) discussion of the symptoms of brand canker caused by *Coniothyrium wernsdorffiae*, he stated that the majority of cankers were found around the dormant buds. This is an especially noticeable symptom of the disease in the United States. Of the many cankers examined, the majority appeared to have had their origin in the vicinity of a dormant bud. A few, however, originated without a wound or a bud as the locus of infection.

The results of the inoculation experiments in the present study indicate that the fungus is capable of infecting dormant buds. Growth was more rapid, however, when the germ tubes were able to gain entrance through wounds.

STEM CANKER AND GRAFT CANKER

In Vogel's discussion of the rose-graft disease (*22*) his conclusions as to the identity of the causal fungus were based upon his inoculation experiments, the size of the spores and pycnidia, and the comparison of cultures of the organism found on rose with those of *Coniothyrium fuckelii* isolated from black-raspberry canes. He stated that "a characteristic symptom of this disease is the occurrence of lesions on the scion at the union, and just above the union." In the present study, therefore, only those specimens which showed definite cankers at the union of stock and scion were considered as affected with the graft disease. The fungus causing these cankers was closely compared with that occurring on the so-called stem cankers. Therefore the symptoms, morphology, and physiology of these two fungi will be considered together.

SYMPTOMS

In the case of well-developed graft cankers, the color is darker than that of stem cankers. It varies from Dresden or cinnamon brown [10] at the center to hazel or auburn at the margin. The main portion of the stem cankers, however, varies in color from wood brown or cinnamon buff to snuff brown or Saccardo's umber. The margin is frequently army brown.

In both cases the presence of the fungus can be detected by means of a hand lens because of the sooty covering of spores over the slightly erumpent pustules. A splitting of the bark along the margin of the canker and the succeeding callus formation are characteristic of both of these diseases.

MORPHOLOGY OF THE CAUSAL FUNGI

Mycelium

The mycelia in the tissue of the two types of cankers appeared to be similar. No distinguishing features could be observed. This also was true of the mycelia developed in cultures. On corn-meal agar the mycelia were comparatively scanty and were white without any conspicuous tinge of gray. The gray was more pronounced, however, on the dextrose and synthetic agars, on which also the aerial mycelia were luxuriant. The mycelia on malt agar were not so luxuriant and were a slightly darker gray. On potato agar and sterilized potato slices the mycelia were very luxuriant and the color varied from white to light gray.

Formation of Pycnidia and Spores

The pycnidia and spores of both the graft-canker and stem-canker fungi were formed similarly to those of the brand-canker fungus. In the case of the graft-canker fungus, however, the majority of the pycnidia on the host were simple globose unilocular structures. (Fig. 9.) The stem-canker fungus appeared to form complex pluriloculate pycnidia arising from several confluent primordia. (Fig. 10.) In this respect it resembled the brand-canker fungus.

In Saccardo's description of *Coniothyrium fuckelii* (*19*) he stated that the papillate ostiole is scarcely prominent. Thomé (*21*) described the pycnidia of the same fungus as having an inconspicuous, scarcely prominent papillate ostiole. The original description of *C. rosarum* by Cooke and Harkness (*2*) did not mention the type of ostiole or the method of spore discharge. In the diagram given by Vogel (*22*) the spores of his *C. rosarum* appear to be exuding through a pore in the upper wall of the pycnidium. This method of spore discharge would correspond with that observed by Archer (*1, p. 51*) in his study of *C. concentricum*. He stated that "no definite ostiole is formed but it seems that usually there is merely a rupture or dissolution in the upper wall, at a point just below a stoma, which allows for the discharge of the spores."

A papillate ostiole was found in the pycnidia of both the fungi studied. The manner of its formation and its general character were similar. A buffer tissue was developed, as in the pycnidia of the brand-canker fungus, with an ostiolate canal and periphyses. The buffer tissue was not so conspicuous, however, and both buffer tissue

[10] Ridgway, R. Op. cit.

and periphyses became disorganized when the pycnidia reached maturity and thus eventually disappeared. The ostiolate canal was very short, and, as Thomé (*21*) stated, the papilla scarcely breaks through the epidermis.

In culture the pycnidia were developed in much the same manner as on the host. No buffer tissue, however, was found in connection with either fungus. The first pycnidia formed were usually simple uniloculate structures. Later both fungi developed complex pluriloculate pycnidia arising from confluent primordia. Vogel (*22*) stated that in culture the pycnidia of his *Coniothyrium rosarum* were larger than those of his *C. fuckelii* isolated from black raspberry. It is possible that he was comparing the complex pycnidia of one fungus with the simple pycnidia of the other.

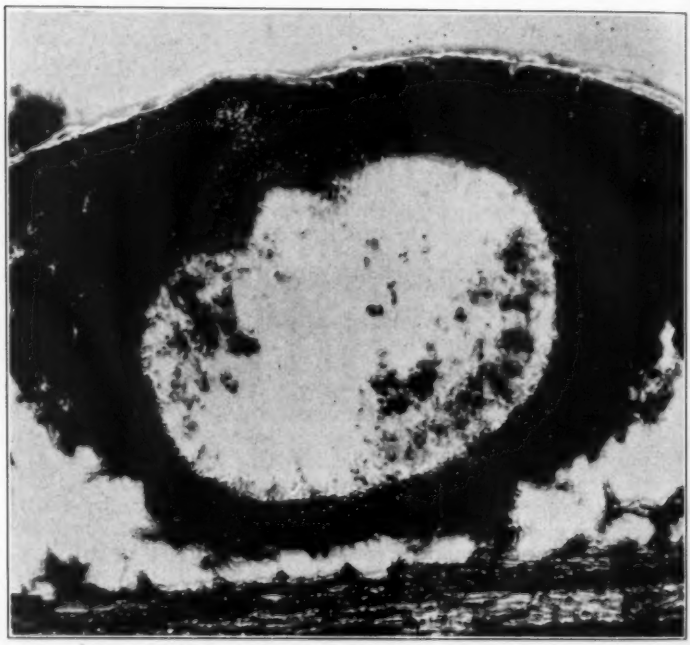

FIGURE 9.—A simple unilocular pycnidium of the graft-canker fungus. × 200

Saccardo (*19*) described *Coniothyrium fuckelii* as "basidiis non visibilibus." Thomé (*21*), in his description of the same fungus, stated that the conidiophores are not perceptible. No mention of the conidiophores is made by Cooke and Harkness (*2*) in connection with *C. rosarum*. Vogel also (*22*) does not mention them, and from the diagram included in his article it would appear that he failed to find them.

The spores obtained from the two types of cankers studied by the writer were formed by budding, in exactly the same manner as those of the brand-canker fungus. No evidence of an endogenous spore origin could be found.

Size and Shape of Spores

Vogel (*22*), in the discussion of his *Coniothyrium rosarum*, distinguished this species from *C. fuckelii* as follows:

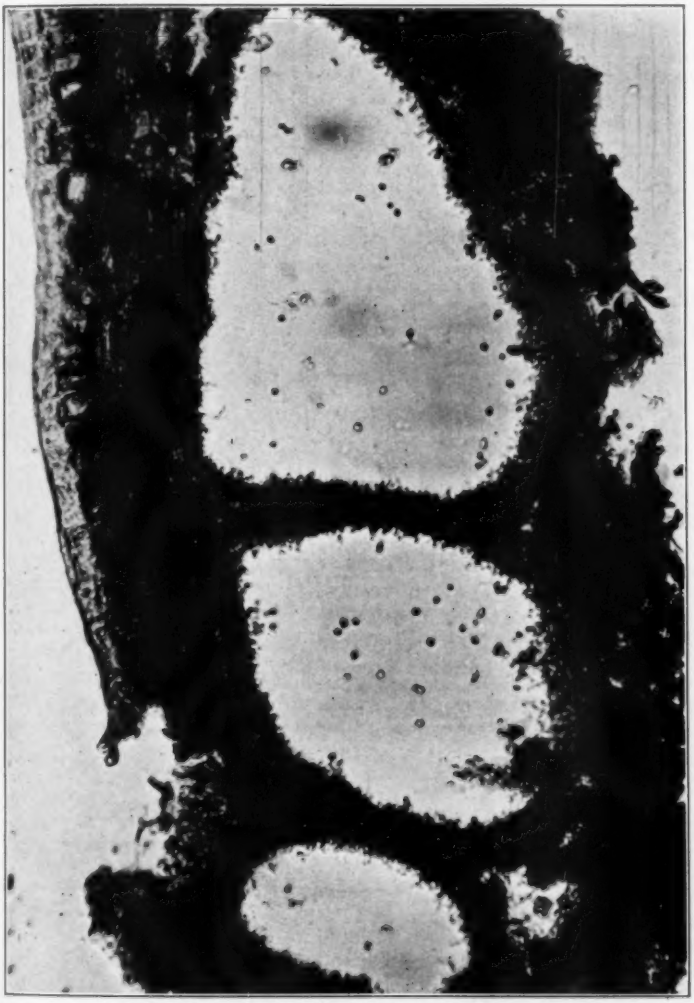

FIGURE 10.—Aggregate pycnidia of the stem-canker fungus. × 160

The spores [*C. rosarum*] are single celled, globose to ellipsoid, brown, 3μ by 3μ to 4μ in size. These measurements are the same as given by Cooke and Harkness for *Coniothyrium rosarum*. * * *

The measurements of *C. fuckelii* given by Massee are, pycnidia 180μ to 200μ in diameter; spores 3μ to 4μ by 2μ to 3μ.

Saccardo (*19*) reported the spore measurements of *Coniothyrium fuckelii* as 2.4μ to 5μ by 2μ to 3.5μ, and of the variety of *C. fuckelii* on Rosa as 3μ to 5μ by 3μ. The description of *C. fuckelii* given by O'Gara (*16*) corresponds with that by Saccardo except for the spore measurements, which he gave as 2μ to 4.5μ by 2μ to 3.5μ. The difference in the measurements given for spore length might be explained as the result of an accidental transposition of the decimal point and the figure 4.

It was impossible in the present study to formulate a plan for measuring the spores produced by the fungi from the two types of cankers which would eliminate from consideration the modifying influence of all contributory environmental factors. The fact that the so-called graft canker is produced typically on young grafts under greenhouse conditions and the stem canker on older plants in either the greenhouse or the garden creates difficulties in making a satisfactory comparison. Moreover, the smallness of the spores and the difficulty of selecting for measurement spores of equivalent maturity add to the possibility of inaccuracies in the calculation and comparison of spore size. For these reasons it is evident that the data on spore measurements here presented do not constitute a comprehensive biometric study, but show the results obtained from certain specimens grown under the conditions stated.

The spores were measured according to the procedure already described for the spores of the brand-canker fungus. In order to increase visibility, a stain consisting of 10 gm. of erythrosin was added to the mounting medium. The source of the material and the results obtained are given in Table 1, and the ratio of length to width in Table 2.

In the actual measurements the spores were found to vary considerably in both length and width. The actual range of the spores was as follows: Graft-canker fungus, 2.0μ to 4.0μ by 1.6μ to 3.6μ; stem-canker fungus, 2.0μ to 4.4μ by 1.2μ to 3.0μ. It is apparent that these measurements, respectively, show the same range as those of Cooke and Harkness for *Coniothyrium rosarum* and of Massee for *C. fuckelii*. As in the case of the brand-canker fungus, spores larger than those of the ranges given were exceedingly rare and were usually found only in mounts bearing germinating spores.

PHYSIOLOGY OF THE CAUSAL FUNGI

Description of Cultures on Various Media

Comparative series of cultures were made of the fungi isolated from the two types of cankers. Specimens of the cankers were placed in moist chambers, and as soon as the spores exuded from the pycnidia in a gelatinous mass a few of them were transferred to the culture tubes. Transfers were always made on the same day and under conditions of environment as similar as possible. As previously stated, no distinguishable differences could be detected between the organisms from the two types of cankers in either the mycelial growth or the production of pycnidia. The characteristics of growth upon the various media were as follows:

Malt agar.—The young mycelium was almost white, that is, just barely tinged with gray. As development proceeded the aerial mycelium became

moderately abundant and fluffy with a more decided grayish tinge. The young advancing mycelium, however, appeared white. In about two weeks after inoculation the pycnidia began to develop. They were produced in moderate abundance and were usually single. In an old culture, however, the tendency toward aggregate pycnidia was noticeable.

CORN-MEAL AGAR.—A small amount of white mycelium was produced, which was more closely matted than on malt agar and showed no conspicuous tinge of gray. Pycnidia were developed in abundance and were at first a yellowish brown, deepening to dark brown at maturity. The black sooty mass of spores at the ostiole was particularly noticeable. The rate of growth was relatively the same as on malt agar.

POTATO AGAR.—The mycelia in all the cultures on potato agar were white or very light gray and moderately abundant. Numerous dark-brown fruiting bodies were formed. The rate of growth was slightly more rapid than on the other media.

STERILIZED POTATO SLICES.—This medium proved very satisfactory for the production of both mycelium and fruiting bodies. Dense white aerial mycelia and complex pycnidia were formed in all the cultures.

DEXTROSE AGAR.—As in the case of the brand-canker fungus, the mycelial growth was medium in amount. Comparatively few pycnidia were formed in the grayish-white mycelium.

SYNTHETIC AGAR.—Excellent cultures resulted from the use of this medium. The mycelium was abundant, fluffy, and white slightly tinged with gray. Good-sized pycnidia were produced in moderate amount, both single and aggregate structures being found.

ROSE-STEM AGAR.—The organism produced only a moderate growth; a few pycnidia being developed to maturity.

STERILIZED ROSE STEMS.—Portions of rose stems were prepared as already described in connection with the brand-canker fungus, and were inoculated with spores, mycelium, or pycnidia of the organisms from the graft cankers and the stem cankers. This method was the most successful for obtaining an abundance of mature pycnidia and spores.

SPORE GERMINATION

Germination experiments, comparable to those for the brand-canker fungus, were made in tap water, distilled water, and the various media. The spores from both cankers germinated readily after 24 hours in tap water, but not quite so readily in distilled water. Moreover, the percentage of germination was greater in tap water than in distilled water. On the agars used the rate and the percentage of germination gave results similar to those from the hanging-drop cultures in tap water. In all cases a pronounced swelling of the spore preceded germination. (Fig. 11.) Septation of the spores, which was a characteristic of the spores of the brand-canker fungus, occurred only rarely.

PATHOGENICITY OF THE ORGANISMS

INOCULATION EXPERIMENTS

Rose cuttings grown in pots of sterilized sand were inoculated as in the experiments conducted with the brand-canker fungus. In all cases cankers were produced on both the cuttings inoculated in the axil of a bud and those inoculated through wounds. The fungus was reisolated from the diseased areas. The pycnidia produced on the cankers showed both simple and complex structures.

Cuttings of red-raspberry canes were similarly inoculated, and cankers resulted. The pycnidia differed from those produced on the rose in that they showed a tendency to be single and unilocular rather than complex plurilocular.

Inoculations of leaves in moist chambers proved unsuccessful. Since a collection of specimens of hips from hybrid plants of *Rosa rugosa* showed an infection by a fungus resembling *Coniothyrium fuckelii*, a number of healthy hips were inoculated in moist chambers with the spores of the fungi isolated from the graft cankers and the stem cankers. Pycnidia and spores were readily produced on the inoculated hips in all cases. (Fig. 12.)

Penetration of the Host

From the results of the inoculations on the rose cuttings it is evident that infection may take place through either dormant buds or wounds. An examination of many specimens of stem canker in

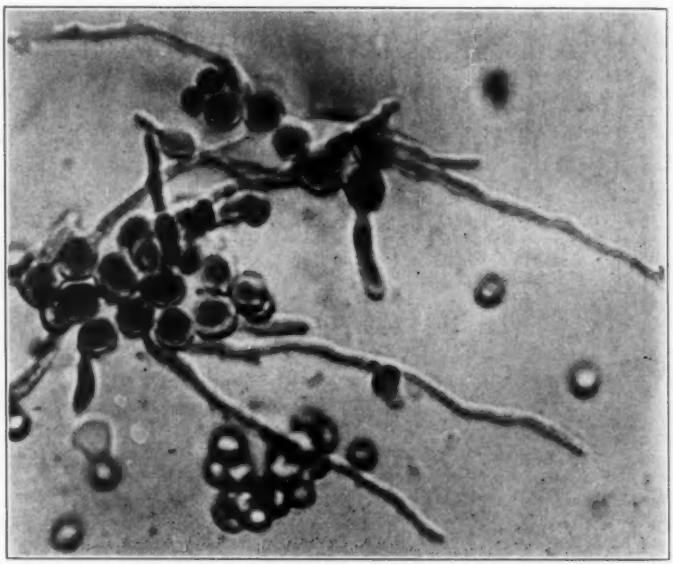

Figure 11.—Germinating spores of the graft-canker fungus. × 1,300

nature leads to the conclusion that the germ tubes are capable of entering the tissue even if no wounds or buds are present. Such infection, however, seems to be extremely rare. The diseased areas usually occur around wounds such as those caused by the breaking or pruning of stems, by the rubbing of the prickles of one stem against the bark of another, by the accidental removal of the prickles, or by insects. Rarely does one find a pruned stem which does not have a few pycnidia on the cut end at the inner margin of the bark. The same condition is very frequently found on rose cuttings, particularly those made from a plant affected with stem canker. Graft cankers also appear to result from infection through wounds or through the callus at the point of union of stock and scion. In the present study it was found that the fungus may also infect through dormant buds.

CONCLUSIONS

It is evident from this study of the morphology and physiology of the fungi isolated from the three types of cankers that the brand-canker fungus is a different species of Coniothyrium from that causing the so-called stem cankers and graft cankers. This corroborates the statements made by Laubert (*10*) and by Jenkins and Martin (*8*), who attributed the disease to *Coniothyrium wernsdorffiae*. In the case of the fungi isolated from stem canker and graft canker there was no evidence of any morphological or physiological differences between them. Since the fungus obtained in cultures from the stem cankers was similar to that produced in cultures from spores of *Leptosphaeria coniothyrium*, the writer felt justified in considering it to be *C. fuckelii*. The results of the present study would seem to indicate, therefore, that the graft cankers were produced by the same fungus that caused the stem cankers. The graft-canker fungus, moreover, resembled that collected and identified by Vogel (*22*) as *C. rosarum* in so far as its morphology under natural conditions is concerned. Unfortunately, type specimens of *C. rosarum* were not available for examination. Regardless of this fact, however, the conclusion may be drawn that the two types of cankers are caused by one and the same fungus, which resembles the imperfect stage of *L. coniothyrium* described by Saccardo as *C. fuckelii*.

FIGURE 12.—A rose hip infected with the stem-canker fungus, showing the concentric formation of the pycnidia on the infected spot

SUMMARY

Three diseases attributed to species of Coniothyrium have been described as occurring upon the genus Rosa in the United States. Because of the similarity of the symptoms of the diseases, there has been considerable doubt as to the identity of the causal fungi. The present study was undertaken to determine what morphological and physiological characters might be of value in diagnosing the diseases.

The fungus isolated from the so-called brand-canker disease may be distinguished from other species of Coniothyrium on rose by the pronounced buffer tissue and papilla as well as the long ostiolar canal of its pycnidia, the size of its spores, and the grayish color of its mycelium in culture. Spore germination and the rate of growth in culture were relatively slow. The spores of the fungus were produced by budding from the layer of cells lining the pycnidial cavity. Inoculation experiments showed that the fungus was capable of infecting rose and red-raspberry cuttings through dormant buds and

through wounds. Inoculations of rose leaves and hips were not successful. The fungus is designated as *Coniothyrium wernsdorffiae* Laub.

No morphological or physiological differences could be detected between the fungi isolated from the so-called stem cankers and graft cankers. The pycnidia varied from simple uniloculate to complex pluriloculate structures and developed an inconspicuous buffer tissue and papillate ostiole. The mycelium in culture was white or light gray. The spores were produced by budding, as in *Coniothyrium wernsdorffiae*. Rose and red-raspberry cuttings were successfully inoculated through dormant buds and through wounds, with the production of cankers. Rose hips became readily infected following inoculation, but no growth occurred on rose leaves. The results of this study seem to indicate that the stem cankers and graft cankers were caused by the same species of Coniothyrium, which is designated as *C. fuckelii* Sacc.

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INHERITANCE OF CERTAIN SEED-COAT COLORS IN SOYBEANS [1]

By RALPH T. STEWART [2]

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INTRODUCTION

Since varieties and strains of soybeans (*Soja max*) are largely identified by the color of their seed coats, it is of no small consequence to the plant breeder to learn as much as possible about the inheritance of the different color types and patterns. Some work on this problem had been published by different investigators prior to 1923 when the writer took up this study. Since then other investigators have published their findings on seed-coat inheritance in soybeans. The results obtained by the various workers have, however, been rather conflicting and in some cases have been based on few data largely obtained in the F_2 generation. It seems, then, that additional data on the inheritance of seed-coat color in soybeans should be of value in helping to clear up the problem.

In taking up this study the writer originally planned to use several varieties, to make a large number of crosses between them, and to study progenies from these crosses through the F_2 and F_3 generations, but owing to the extreme difficulty encountered in hybridizing soybeans the original plans were to a considerable extent abandoned. However, during the progress of the work the writer has been able to make a rather extensive study of a few crosses which involve some interesting genetic relationships between certain pigment colors in the seed coat. Most of these relationships have been reported by previous workers, and although their findings and symbols are somewhat different, their work contributes much to the solution of the problem. The results of all previous genetic work with soybeans have been summarized by Owen (*5*)[4] and need not be taken up here. Closely related work will, however, be discussed whenever it has a direct bearing on the problem in hand.

MATERIALS AND METHODS

In this study only varieties of soybeans with yellow, black, and brown pigments in their seed coats have been used. No crosses involving green seed-coat pigments were available. Most of the data used in this study were obtained from progenies of F_1 seeds of crosses turned over to the writer in the spring of 1923 by John B. Wentz, of the department of farm crops and soils, Iowa State College.

[1] Received for publication June 20, 1929; issued May, 1930. These investigations were conducted at Iowa State College, Ames, Iowa.

[2] The writer acknowledges the many suggestions and untiring assistance rendered by Dr. John B. Wentz in planning and bringing this problem to completion. He also wishes to extend thanks to Prof. H. D. Hughes and F. S. Wilkins for freely lending equipment necessary to carry on the work. Dr. E. W. Lindstrom and Prof. W. V. Lambert have given helpful suggestions in the preparation of the thesis.

[3] Now associate professor of agronomy, Agricultural and Mechanical College of Texas.

[4] Reference is made by number (italic) to "Literature cited," p. 854.

Since that time the writer has added two crosses and two natural hybrids.

In this investigation the progenies of 13 different crosses involving 8 different combinations of varieties and 2 natural hybrids have been studied. During the course of this work the writer has been able to observe the genetic behavior of most of the crosses through the F_3 generation. One family in cross No. 5 has been grown through the F_4 generation.

In order to avoid as far as possible any mixing or contamination, seeds from the F_1 and F_2 plants were harvested and threshed by hand. At the time of harvesting, the seeds from each plant were put into separate envelopes and the envelopes numbered according to the row and plant number. All plants of progenies segregating for pubescence color were tagged several weeks before harvesting in order that pubescence and seed-coat relationships could be studied. The F_3 plants were pulled by hand and tied into bundles. Individual plants were threshed with a small threshing machine and the seeds from each plant put into a numbered envelope.

INHERITANCE OF HILUM COLOR

Woodworth (7) reported data on the inheritance of black and brown hilum color. Black hilum color was reported as being dominant to brown. To explain his data, it was necessary to assume two factors, *B* and *H*. When both factors were present in the zygote the hilum color was black. If either *B* or *H* was absent, the hilum color was brown. Thus a 9 to 7 interaction of factors was established. Individuals without either *B* or *H*, but containing their recessive allelomorphs, *b* and *h*, could not be distinguished from those having only one of the dominant factors. In all other cases thus far reported the inheritance of hilum color has been explained by assuming a single-factor difference.

In the present studies two crosses, No. 113 and No. 527, which involve different hilum colors, were obtained. Data for these crosses are presented in Table 1.

TABLE 1.—*F_2 progenies from crosses between varieties of soybeans having brown and black hilums, Ito San × Manchu*

Cross No.	Black hilum	Brown hilum	Deviation 3:1	Deviation P. E.
113–1	56	17	1.25	0.50
113–2	61	16	3.25	1.27
527	9	4	.75	.71
Total	126	37	3.75	1.00

The male parent was a selection from the Ito San variety and the female parent a selection from the Manchu variety. Both parents for these crosses have yellow seed coats, but the male parent has a brown hilum and the female a black hilum. Two F_1 plants were obtained from cross No. 113 and one F_1 plant from cross No. 527. Pure lines of both parents bore seeds which were extremely mottled, the color of the mottling always being the same as the color of the

hilum. Seeds borne on the F_1 plants were yellow with black hilums and black mottling over the sides. In the F_2 generation, plants bearing seeds with black hilums and plants bearing seeds with brown hilums appeared in approximately a 3:1 ratio. From the 2 F_1 plants in cross No. 113, 117 F_2 plants bearing seeds with black hilums and 33 plants bearing seeds with brown hilums were obtained. The deviation from a 3:1 ratio in this cross was 4.50 ± 3.58. Only 13 F_2 plants were obtained from cross No. 527. Of these, 9 bore yellow seeds with black hilums and 4 yellow seeds with brown hilums. In this cross the deviation from a 3:1 ratio was 0.75 ± 1.05.

When both crosses are considered together, 126 of the F_2 plants bore yellow seeds with black hilums and 37 yellow seeds with brown hilums. The deviation from a 3:1 ratio with all plants considered is 3.75 ± 3.75. This difference can well be attributed to chance, and one factor pair suffices to explain the data.

In these crosses pigment in the hilum is apparently due to the same factors as in the seed coat, and since the author is using the factors adopted by Owen (*5*) for seed-coat color, the factor pair R_1r_1 is used instead of *Bb* used by Woodworth.

INHERITANCE OF SEED-COAT COLORS

In these studies crosses have been made between varieties which involve different color combinations of the seed coat. In order to present the results more clearly it is thought advisable to give the data for these different color combinations separately.

CROSSES INVOLVING LIGHT-BROWN AND REDDISH-BROWN SEED COATS

Nagai (*1*) has reported results of crosses between light-brown and reddish-brown varieties of soybeans. His data show that reddish-brown is recessive to the lighter brown color. A single-factor pair, *Oo*, is responsible for the difference.

The writer has been able to gather considerable data on the inheritance of these two colors from reciprocal crosses between the Ogemaw and Soysota varieties. The Ogemaw variety has a reddish-brown seed coat with a more or less dull appearance. The Soysota variety, on the other hand, has a light-brown seed coat with a more or less glossy appearance. F_1 seeds from these crosses, regardless of the way the crosses were made, were more or less intermediate between the two parents in color. However, they more nearly approach the shade of the lighter-colored parent. The F_2 plants appeared to show the three classes—light, intermediate, and reddish-brown colors—suggesting incomplete dominance. The F_2 plants were classified into the three classes, although considerable difficulty was experienced in separating the light-brown from the intermediate class. No difficulty was experienced, however, in separating the reddish-brown class.

A larger number of the F_2 plants were tested in the F_3 generation. The seed-coat colors observed in the F_3 generation showed that many of the F_2 plants classified as light brown were heterozygous; also that many of the F_2 plants classified as intermediate failed to segregate as expected for the reddish-brown color. Thus, the F_3 results showed the fallacy of trying to separate the light-brown from the intermediate class. With a reclassification—that is, grouping the light and intermediate classes together—there were obtained from all of the crosses

between these two varieties 472 F_2 plants with light-brown seeds and 149 with reddish-brown seeds. (Table 2.) The deviation from the expected 3:1 ratio was 6.25 ± 7.28, confirming a single-factor difference between the light-brown and reddish-brown pigment colors.

The symbol r_1 has been used by Owen to designate the factor for the light-brown pigment of the Soysota variety, and *o* has been used by Nagai to designate the factor for reddish-brown pigment of the Ogemaw variety. Following the nomenclature of both of these authors, r_1 is used as the factor for light brown and $r_1{}^o$ the factor for reddish brown. Data for the F_2 plants are presented in Table 2.

TABLE 2.—*F_2 progenies from reciprocal crosses between varieties of soybeans having light-brown and reddish-brown seed coats*

Cross	Light brown	Reddish brown	Deviation 3:1	Deviation / P. E.
Soysota×Ogemaw:				
No. 50–1	61	22	1.25	0.47
No. 50–2	63	22	.75	.29
Ogemaw×Soysota:				
No. 64–1	62	23	1.75	.66
No. 64–2	65	25	2.50	.90
No. 65–1	51	15	1.50	.63
No. 65–2	63	11	7.50	2.99
No. 73–1	54	14	3.00	1.24
No. 73–2	53	17	.50	.20
Total	472	149	6.25	.86

TABLE 3.—*Assumed genotypes together with breeding behavior of F_2 plants when tested in the F_3 generation*

[Cross No. 50 Soysota (*ii* r_1r_1 R_2R_2) × Ogemaw (*ii* $r_1{}^or_1{}^o$ R_2R_2]

Genotype and F_2 plant No.	Phenotypes observed in the F_3 generation [a]		Genotype and F_2 plant No.	Phenotypes observed in the F_3 generation [a]		Genotype and F_2 plant No.	Phenotypes observed in the F_3 generation [a]	
	Light brown	Reddish brown		Light brown	Reddish brown		Light brown	Reddish brown
ii r_1r_1 R_2R_2:			*ii* $r_1{}^or_1{}^o$ R_2R_2—Contd.			*ii* $r_1r_1{}^o$ R_2R_2—Contd.		
1–4	14		1–40		42	1–33	20	8
1–5	40		1–44		36	1–34	38	7
1–6	40		1–48		40	1–35	29	7
1–8	53		1–49		12	1–36	29	12
1–11	49		1–50		39	1–38	21	8
1–12	31		1–54		29	1–41	27	4
1–15	21		1–55		29	1–42	37	12
1–16	38		*ii* $r_1r_1{}^o$ R_2R_2:			1–45	34	5
1–21	44		1–1	35	13	1–46	22	6
1–30	36		1–2	42	12	1–51	27	5
1–37	14		1–3	33	13	1–52	13	2
1–47	34		1–7	9	2	1–53	39	12
1–58	52		1–13	30	17	1–56	29	9
1–61	45		1–14	28	9	1–57	32	6
1–88	23		1–18	33	13	1–59	11	2
ii $r_1{}^or_1{}^o$ R_2R_2:			1–23	16	9	1–60	30	15
1–9		42	1–26	38	5	1–86	28	12
1–20		51	1–27	21	15	1–87	23	12
1–22		50	1–28	27	8	Total	854	273
1–24		8	1–29	23	4			
1–32		52	1–31	30	9			
1–39		47						

[a] $\frac{\text{Deviation}}{\text{P. E.}} = 0.89$

From cross No. 50 there were 59 F_2 plants tested; from cross No. 64, 97; and from cross No. 65, 147, making a total of 303 F_2 plants tested in the F_3 generation. Of these, 60 were classified as having reddish-brown seed coats and in the F_3 generation bred true. The remaining 243 were classified as having light-brown seed coats. On the basis of a single-factor hypothesis, 81 of the 243 F_2 plants classed as having light-brown seed coats should breed true and 162 should segregate again for 3 light-brown to 1 reddish-brown. The actual counts were 81 breeding true and 162 segregating, an exact fit. From the 81 F_2 plants showing no segregation 2,771 F_3 plants were grown. From the 162 F_2 plants which segregated, 6,192 F_3 plants were obtained. Of these, 4,688 were light brown and 1,504 were reddish brown. The deviation from the expected 3:1 ratio is 44.00 ± 20.00. The F_3 data for the above crosses are presented in Tables 3, 4, and 5.

TABLE 4.—*Assumed genotype together with breeding behavior of F_2 plants tested in the F_3 generation*

[Cross No. 64 Ogemaw (*ii* $r_1{}^c r_1{}^c$ R_1R_2)×Soysota (*ii* r_1r_1 R_1R_2)]

Genotypes and F_2 plant No.	Phenotypes observed in the F_3 generation [a]		Genotypes and F_2 plant No.	Phenotypes observed in the F_3 generation [a]		Genotypes and F_2 plant No.	Phenotypes observed in the F_3 generation [a]	
	Light brown	Reddish brown		Light brown	Reddish brown		Light brown	Reddish brown
ii $r_1r_1R_1R_2$:			*ii* $r_1{}^c r_1{}^c R_1R_2$—Contd.			*ii* $r_1r_1{}^c R_1R_2$—Contd.		
1-8	25		2-50		44	2-35	39	11
1-10	49		2-51		29	2-36	37	11
1-13	8		2-56		41	2-37	34	15
1-17	6		2-65		53	2-38	44	7
2-18	25		2-68		42	2-46	19	6
2-20	37		2-82		44	2-47	32	10
2-21	53		2-89		51	2-49	29	11
2-28	43		2-91		2	2-52	26	9
2-34	42		2-94		50	2-54	41	14
2-39	43		2-95		43	2-55	38	10
2-45	18		*ii* $r_1r_1{}^c R_1R_2$:			2-57	32	20
2-53	46		1-1	21	11	2-58	21	3
2-59	5		1-2	39	13	2-60	22	14
2-61	45		1-3	40	7	2-63	32	16
2-62	39		1-4	28	6	2-67	39	9
2-64	45		1-6	39	8	2-69	21	8
2-66	40		1-7	24	9	2-70	24	4
2-73	47		1-12	42	16	2-71	46	5
2-77	43		1-14	23	3	2-72	23	16
2-81	33		1-18	30	15	2-74	37	11
2-83	6		2-12	11	2	2-75	37	9
2-88	30		2-13	32	8	2-76	5	6
2-90	33		2-14	33	7	2-78	31	10
ii $r_1{}^c r_1{}^c R_2R_2$:			2-15	19	6	2-79	28	4
1-5		38	2-16	31	9	2-80	33	14
1-11		33	2-19	34	10	2-84	31	7
1-15		48	2-22	36	12	2-86	38	9
1-16		48	2-23	23	8	2-87	34	9
2-17		34	2-24	34	8	2-92	25	8
2-29		47	2-25	23	10	2-93	24	8
2-33		38	2-27	17	7			
2-40		48	2-30	31	13	Total	1,575	497
2-42		35	2-31	33	10			
2-44		28	2-32	10	5			
2-48		47						

[a] $\frac{\text{Deviation}}{\text{P. E.}} = 1.58$.

TABLE 5.—*Assumed genotypes together with breeding behavior of F_2 plants when tested in the F_3 generation*

[Cross No. 65 Ogemaw (*ii* $r_1{}^\circ r_1{}^\circ$ R_2R_2) × Soysota (*ii* r_1r_1 R_2R_2)]

F_2 genotype and F_2 plant No.	Phenotypes observed in the F_3 generation [a]		F_2 genotype and F_2 plant No.	Phenotypes observed in the F_3 generation [a]		F_2 genotype and F_2 plant No.	Phenotypes observed in the F_3 generation [a]	
	Light brown	Reddish brown		Light brown	Reddish brown		Light brown	Reddish brown
ii r_1r_1 R_2R_2:			*ii* $r_1{}^\circ r_1{}^\circ$ R_2R_2—Contd.			*ii* $r_1r_1{}^\circ$ R_2R_2—Contd.		
1-5	46		1-44		40	1-65	37	5
1-8	45		1-49		24	1-67	23	15
1-9	30		1-55		20	1-68	27	21
1-17	38		1-56		41	1-70	22	6
1-22	42		1-58		22	1-73	31	14
1-25	47		1-59		18	1-74	35	11
1-28	48		1-61		36	1-75	30	10
1-31	37		1-63		43	1-76	26	14
1-34	44		1-66		46	1-79	14	6
1-41	31		1-77		29	1-80	28	5
1-46	46		1-78		12	2-1	23	10
1-50	44		2-21		39	2-3	34	9
1-51	20		2-22		39	2-5	36	12
1-54	34		2-28		20	2-9	37	12
1-65	26		2-38		40	2-10	31	8
1-62	23		2-43		36	2-12	32	13
1-71	22		2-65		3	2-15	24	8
1-72	24		2-67		31	2-17	32	7
2-2	47		2-70		34	2-18	34	8
2-4	28		*ii* $r_1r_1{}^\circ$ R_2R_2:			2-20	34	11
2-6	23		1-1	31	9	2-25	29	10
2-7	52		1-2	38	8	2-26	42	9
2-11	17		1-3	38	9	2-27	28	8
2-14	22		1-4	31	17	2-29	23	7
2-16	48		1-6	31	14	2-33	28	12
2-19	13		1-7	43	11	2-34	37	11
2-23	25		1-11	35	6	2-35	29	17
2-24	3		1-13	29	10	2-36	34	24
2-32	21		1-14	31	14	2-39	11	3
2-37	46		1-18	5	8	2-41	28	15
2-40	33		1-19	30	9	2-43	25	4
2-45	35		1-20	38	11	2-44	41	11
2-48	44		1-24	29	10	2-46	15	4
2-50	25		1-29	33	9	2-47	26	9
2-51	44		1-30	33	12	2-49	36	11
2-56	39		1-35	20	8	2-52	31	11
2-58	34		1-36	22	8	2-53	35	8
2-68	34		1-37	28	6	2-54	35	3
2-72	48		1-38	33	7	2-55	30	15
2-73	30		1-39	31	10	2-61	12	3
2-74	49		1-40	25	4	2-62	12	3
2-75	23		1-42	23	11	2-63	31	11
2-76	46		1-43	31	10	2-64	34	5
ii $r_1{}^\circ r_1{}^\circ$ R_2R_2:			1-45	34	11	2-66	34	7
1-10		42	1-47	28	10	2-69	13	5
1-12		45	1-48	33	4	2-71	30	6
1-15		32	1-52	9	4	2-77	33	16
1-16		19	1-53	7	3	2-78	47	8
1-21		40	1-57	35	9			
1-26		49	1-64	26	11	Total	2,259	734
1-33		40						

[a] $\frac{\text{Deviation}}{\text{P. E.}} = 0.89$.

CROSS INVOLVING VARIETIES WITH BLACK AND BROWN SEED COATS

Piper and Morse (*6*) reported black pigment to be dominant to brown. A single-factor difference explained their data. Owen (*5*) reported on the inheritance of black and brown seed-coat colors. In one case his data conform to the findings of Piper and Morse. In this cross black was found to be fully dominant to brown, and the assumption of a single-factor difference sufficed to explain his data.

In the second case the behavior was found to be quite different. Data taken from a natural hybrid showed incomplete dominance of black over brown. An intermediate class which was black and speckled with brown was observed. Further breeding showed these intermediate classes to be heterozygous.

The writer has been able to obtain but one cross involving these two colors, that is, cross No. 502 (Wisconsin Black × Soysota). Seeds borne on the F_1 plant were fully black. Only 13 F_2 plants were obtained. Of these, 9 were black and 4 were brown. The deviation from the expectant 3 : 1 ratio was 0.75 ± 1.05. The results can, therefore, be explained on the basis of a single pair of factors, black being dominant over brown. The factors R_1r_1 proposed by Owen are used to designate this difference.

CROSSES INVOLVING VARIETIES HAVING YELLOW AND BLACK SEED COATS

Data on three crosses between varieties having yellow and black seed coats have been obtained. Cross No. 206 (Manchu × Wisconsin Black) and cross No. 407 (Manchu × Wilson) are very similar and may be discussed together. In these two crosses the female parents are from the same variety, Manchu. Pure lines from the female parent of each cross were grown and as far as seed-coat color is concerned could not be distinguished, the seed-coat color being in each case yellow, mottled with black. The male parent in each cross bore black seeds. There was, however, considerable difference in their habit of growth. The Wisconsin Black parent stood erect and was more or less bushy while the parent from the Wilson variety was of a reclining type and showed a tendency to be more or less viny.

The F_1 seeds from both varieties were yellow, mottled with black, similar to the seeds of the pure-line female parents. In the F_2 generation, from cross No. 206, 45 plants were obtained. Of these, 34 were yellow mottled with black, though in varying degrees, and 11 were self black. On the basis of a 3 : 1 ratio, the deviation from the expected was 0.25 ± 1.96. From cross No. 407, 122 plants bearing seeds which were yellow mottled with black and 37 plants bearing seeds which were self black were obtained in the F_2 generation. In this cross the deviation from the expected 3 : 1 ratio was 2.75 ± 3.68. When both crosses were considered together, there were 204 F_2 plants. Of these, 156 bore seeds which were yellow mottled with black and 48 seeds which were self black. The deviation from the expected 3 : 1 ratio was 3.00 ± 4.17. The F_2 data for these crosses are presented in Table 8. Data for the breeding behavior of the F_2 plants are shown in Tables 6 and 7. A single pair of factors will explain the difference, but in this case pigment factors are not involved, since all plants bearing yellow seeds contained black pigment in the hilum. Black pigment also occurred in the seed coat of all mottled beans. The difference then must be in another factor which inhibits or partially inhibits yellow from most of the seed coat. Owen (*6*) has used two such inhibiting factors I^h and I^i which form a multiple allelomorphic series with i to designate this difference. According to his hypothesis a plant homozygous or heterozgyous for either I^h or I^i would bear yellow seeds, and a plant homozygous for their recessive allelomorph, i, would bear self-colored seeds. In these studies the writer was unable to distinguish between I^h and I^i. Therefore, I is used as the factor which inhibits pigment color and the factor pair involved is assumed to be Ii.

TABLE 6.—*Assumed genotypes together with breeding behavior of F_2 plants when tested in the F_3 generation*

[Cross No. 206 Manchu (*II* R_1R_1 R_2R_2)×Wisconsin Black (*ii* R_1R_1 R_2R_2)]

Genotype and F_2 plant No.	Phenotypes observed in the F_3 generation [a]		Genotype and F_2 plant No.	Phenotypes observed in the F_3 generation [a]		Genotype and F_2 plant No.	Phenotypes observed in the F_3 generation [a]	
	Mottled, yellow black	Self-colored black		Mottled, yellow black	Self-colored black		Mottled, yellow black	Self-colored black
II R_1R_1 R_2R_2:			*ii* R_1R_1 R_2R_2—Continued.			*Ii* R_1R_1 R_2R_2—Continued.		
1-3	2		1-20		32	1-27	28	4
1-6	15		1-21		27	1-31	9	6
1-7	15		1-22		25	1-33	25	3
1-10	9		1-28		31	1-35	2	1
1-12	1		1-32		28	1-36	15	3
1-13	4		1-48		19	1-37	12	9
1-19	22		1-43		32	1-38	18	4
1-23	22		*Ii* R_1R_1 R_2R_2:			1-39	10	9
1-26	15		1-1	20	6	1-41	2	1
1-29	20		1-8	10	4	1-42	18	3
1-34	15		1-9	8	5	1-43	33	10
1-50	34		1-15	21	5	1-44	14	8
ii R_1R_1 R_2R_2:			1-17	10	3	1-45	24	3
1-2		16	1-18	17	2	1-46	12	4
1-5		11	1-24	7	4			
1-11		28	1-25	22	4	Total	337	101
1-16		19						

[a] $\frac{\text{Deviation}}{\text{P. E.}}=1.39$.

TABLE 7.—*Assumed genotypes together with breeding behavior of F_2 plants when tested in the F_3 generation*

[Cross No. 407 Manchu (*II* R_1R_1 R_2R_2)×Wilson (*ii* R_1R_1 R_2R_2)]

Genotype and F_2 plant No.	Phenotypes observed in the F_3 generation [a]		Genotype and F_2 plant No.	Phenotypes observed in the F_3 generation [a]		Genotype and F_2 plant No.	Phenotypes observed in the F_3 generation [a]	
	Mottled, yellow black	Self-colored black		Mottled, yellow black	Self-colored black		Mottled, yellow black	Self-colored black
II R_1R_1 R_2R_2:			*ii* R_1R_1 R_2R_2—Continued.			*Ii* R_1R_1 R_2R_2—Continued.		
15	22		22		34	38	25	13
20	21		24		13	42	17	6
21	34		26		20	43	30	6
23	19		31		37	46	14	7
29	29		32		19	52	22	3
39	17		35		38	53	33	3
41	37		44		34	55	13	2
45	17		49		33	57	15	4
48	41		62		36	60	29	8
50	46		64		17	61	32	12
51	30		78		10	63	23	6
54	31		80		27	66	30	8
58	29		89		5	67	17	5
59	26		99		28	68	22	13
65	37		101		38	71	24	10
69	39		102		35	74	25	6
70	36		*Ii* R_1R_1 R_2R_2:			75	28	5
72	24		1	29	10	76	30	10
73	33		2	16	14	77	31	8
79	33		4	30	9	82	31	11
81	25		5	33	9	83	30	7
87	36		8	26	10	84	25	7
88	35		9	20	12	85	28	8
90	35		13	25	7	91	18	9
93	34		14	19	10	92	16	14
97	36		16	25	7	94	24	5
ii R_1R_1 R_2R_2:			18	27	14	95	16	6
3		23	25	20	10	96	9	6
6		25	27	20	10	98	25	9
7		27	28	24	6	100	25	2
10		20	30	11	9	103	26	10
11		37	33	23	7			
12		24	34	21	11	Total	1,122	387
17		36	36	20	3			
19		29						

[a] $\frac{\text{Deviation}}{\text{P. E.}}=0.86$.

In cross No. 5 (Wisconsin Black × Mandarin) quite different results were obtained. Owen (*5*) has reported data from combinations very similar to this cross. In certain of his crosses the Mandarin variety but not the Wisconsin Black was used. Data presented by Owen showed that three factors were involved in his crosses. The phenotypes which are being reported in this study were also reported by Owen, which indicates that the crosses were genetically alike. The female parent of this cross had tawny pubescence and bore self-colored black seeds. The male parent had gray pubescence and bore yellow seeds, mottled with a buff pigment. The F_1 plant had tawny pubescence and produced seeds which were yellow mottled with black. In the F_2 generation 8 different phenotypes were produced in a population of 56 plants, which suggested 3 factors to be involved. The number of plants secured in each of the phenotypes, together with the number expected, are presented in Table 8.

TABLE 8.—*F_2 progenies from crosses between varieties of soybeans having yellow and black seed coats*

Cross	Tawny pubescence				Gray pubescence			
	Mottled		Self-colored		Mottled		Self-colored	
	Yellow black	Yellow brown	Black	Brown	Imperfect black	Buff	Imperfect black	Buff
No. 203, Manchu × Wisconsin Black	34		11					
No. 407, Manchu × Wilson	122		37					
Total observed	156		48					
Deviation 3:1	+3		−3					
No. 5, Wisconsin Black × Mandarin	24	9	6	4	6	2	4	1
Expected	23.6	7.9	7.9	2.6	7.9	2.6	2.6	.9

$\chi^2=2.7$. $P=0.9$.

By classifying the progenies with yellow mottled seeds into one group and those bearing self-colored seeds into another group, the number of plants in each group is 41 and 15, respectively, which approximates a 3 : 1 ratio. The restriction factor *I* for pigment color used in the two preceding crosses can therefore be considered to restrict the pigment in this cross.

Disregarding the yellow color and classifying the F_2 progenies according to the color of pigment in their hilum or seed coat, there are 30 plants which bore seeds showing black pigment, 10 imperfect black, 13 brown, and 3 buff. With two factors involved, the expected ratio is 31.5 : 10.5 : 10.5 : 3.5. The numbers observed conform closely to the expected, and a dihybrid ratio is assumed.

The two factors R_1 and R_2 suggested by Owen (*5*) for black pigment can be used. R_1R_1 produces black pigment, R_1r_2 imperfect black, r_1R_2 brown, and r_1r_2 buff. Plants with the restriction factor *I* have the pigment confined to the hilum or regions around the hilum, and plants with its allelomorph, *i*, have self-colored seeds. By assuming that the three factors act independently of each other the appearance of the eight different phenotypes shown in Table 8 can be explained.

By referring again to Table 8 it may be noticed that the F_2 plants are also segregating for tawny and gray pubescence in approximately

a 3 : 1 ratio, and that all plants with black and brown pigment colors have tawny pubescence and all plants with imperfect black or buff pigments have gray pubescence. Since R_2 is also associated with black and brown pigment and its allelomorph r_2 is associated with imperfect black and buff pigments, it follows that R_2 must also be closely linked with a factor for tawny pubescence and r_2 with the recessive, a factor for gray pubescence. Woodworth (*7*) has designated this factor pair as *Tt*. Owen (*5*) has also used these symbols. They can, therefore, well be used to explain data obtained in these studies. Assuming that the four factors, I, R_1, R_2, and T (R_2 and T showing complete linkage) are involved in this cross, the F_2 data presented in Table 8 can well be explained. The values of χ^2, 2.72, and of P, 0.91 indicate a much better fit than would ordinarily be obtained with only 56 F_2 plants, but the classification is well borne out by the breeding behavior of the F_2 plants shown in Table 9.

TABLE 9.—*Assumed genotypes together with breeding behavior of F_2 plants when tested in the F_3 generation*

[(Cross No. 5 Wisconsin Black (*ii* R_1R_1 R_2T R_2T)×Mandarin (*II* $r_1r_1r_2tr_2t$)]

Genotype and F_2 Plant No.	Phenotypes observed in the F_3 generation								Probability	
	Tawney pubescence				Gray pubescence					
	Mottled		Self-colored		Mottled		Self-colored			
	Yellow black	Yellow brown	Black	Brown	Yellow imperfect black	Yellow buff	Imperfect black	Buff	Deviation / P. E.	χ^2 and P
II R_1R_1 R_2TR_2T:										
8	96									
51	61									
61	64									
II r_1r_1 R_2TR_2T:										
38		93								
ii r_1r_1 R_2TR_2T:										
30				30						
60				57						
II R_1R_1 r_2tr_2t:										
33					35					
II r_1r_1 r_2tr_2t:										
58						31				
ii R_1R_1 r_2tr_2t:										
9							24			
49							25			
62							43			
r_1r_1 r_2tr_2t:										
47								25		
II R_1r_1 R_2TR_2T:										
5	36	16								
25	21	12								
53	20	10								
Total	77	38							2.96	
Ii R_1R_1 R_2TR_2T:										
19	70		20						.90	
II R_1R_1 R_2Tr_2t:										
27	14				5					
28	45				18					
Total	59				23				.95	
II r_1r_1 R_2 Tr_2t:										
3		25				6				
4		19				3				
21		20				7				
Total		64				16			1.53	

TABLE 9.—*Assumed genotypes together with breeding behavior of F_2 plants when tested in the F_3 generation*—Continued

[(Cross No. 5 Wisconsin Black (*ii* R_1R_1 R_2T R_2T)×Mandarin (*II* $r_1r_1r_2r_2t_1t_1$)]

Genotype and F_2 Plant No.	Phenotypes observed in the F^2 generation								Probability	
	Tawney pubescence				Gray pubescence					
	Mottled		Self-colored		Mottled		Self-colored			
	Yellow black	Yellow brown	Black	Brown	Yellow imperfect black	Yellow buff	Imperfect black	Buff	Deviation / P. E.	χ^2 and P
ii R_1r_1 R_2TR_2T:										
6			21	9						
44			40	15						
Total			61	24					1.02	
ii r_1r_1 R_2Tr_2t:										
32			13				5		.40	
ir$_1r_1$ R_2Tr_2t:										
13				81				27	0	
Ii R_1R_1 $r_2t_1r_2t$:										
26					24		7		.46	
Ii r_1r_1 $r_2t_1r_2t$:										
54						42		21	2.26	
ii R_1r_1 $r_2t_1r_2t$:										
16							14	6	.76	
II R_1r_1 R_2Tr_2t:										
1	10	3			3	1				
2	40	13			10	5				
7	51	12			15	6				
20	49	18			13	3				
46	38	6			10	4				
Total	188	52			51	19				2.7, .4
Ii R_1r_1 R_2 TR_2T:										
15	15	5	6	1						
57	36	5	6	6						
Total	51	10	12	7						4.9, .2
Ii R_1R_1 R_2Tr_2t:										
48	12		1		4		2			
52	21		4		4		1			
Total	33		5		8		3			3.1, .4
Ii r_1r_1 R_2Tr_2t:										
31		43		12		9		8		
36		39		15		7		4		
37		29		10		14		5		
40		33		8		8		3		
Total		144		45		38		20		3.1, .4
ii R_1r_1 R_2Tr_2t:										
23			98	21			23	7		
41			13	6			5	1		
45			33	23			7	2		
Total			144	50			35	10		5., 1.2
Ii R_1r_1 $r_2t_1r_2t$:										
17					39	17	9	1		
18					10	3	1	1		
55					36	15	12	2		
Total					85	35	22	4		6.2, .1
Ii R_1r_1 R_2Tr_2t:										
12	33	13	6	1	7	2	3	1		
35	30	10	9	1	4	3	4	0		
50	18	16	6	2	4	1	4	0		
56	5	1	1	1	2	1	1	0		
Total	86	40	22	5	17	7	12	1		15.9, .03

The interaction of the four factors can best be understood by observing the theoretical phenotypic distribution below.

27	$I\ R_1\ R_2\ T$	Yellow, mottled with black—tawny pubescence.
9	$I\ R_1\ r_2\ t$	Yellow, mottled with imperfect black-gray pubescence.
9	$I\ r_1\ R_2\ T$	Yellow, mottled with brown—tawny pubescence.
3	$I\ r_1\ r_2\ t$	Yellow, mottled with buff—gray pubescence.
9	$i\ R_1\ R_2\ T$	Self-black—tawny pubescence.
3	$i\ R_1\ r_2\ t$	Self-imperfect black—gray pubescence.
3	$i\ r_1\ R_2\ T$	Self-brown—tawny pubescence.
1	$i\ r_1\ r_2\ t$	Self-buff—gray pubescence.

Since all classes with R_2 have tawny pubescence, the question at once arises why not assume that T instead of R_2 is the complementary factor to R_1 and that it also causes the development of tawny pubescence? Woodworth (*7*) discussed this point and concluded that such may be the case. Owen (*5*) also has noticed the apparent influence T has on the seed-coat color and assumes that T is identical or completely linked with the C factor described by Nagai (*1*) and Nagai and Saito (*2*) which acts in a complementary way with R_1 to give black color. Just whether T is the only factor concerned or whether another factor, R_2, completely linked with T is responsible for intensifying the anthocyanin pigment to black has not yet been determined. However, it seems that since varieties with black seed coat and gray pubescence are known (Piper and Morse (*6*)), it is more satisfactory to assume a hypothesis based on two factors which here are apparently completely linked but which may occasionally cross over to give rise to the varieties with a black seed coat and gray pubescence.

TABLE 10.—*Genotype of F_2 plants as shown by their breeding behavior in the F_3 generation*

[Cross No. 5 Wisconsin Black ($ii\ R_1R_1\ R_2TR_2T$) × Mandarin ($II\ r_1r_1\ r_2tr_2t$)]

Genotype of F_2 plants	Observed	Calculated	$\frac{(o-c)^2}{c}$	Genotype of F_2 plants	Observed	Calculated	$\frac{(o-c)^2}{c}$
1 $II\ R_1R_1\ R_2TR_2T$	3	0.797	6.089	2 $II\ r_1r_1\ R_2TR_2T$	0	1.594	1.594
2 $II\ R_1R_1\ R_2Tr_2t$	2	1.594	.104	4 $Ii\ r_1r_1\ R_2Tr_2t$	4	3.188	.207
2 $II\ R_1r_1\ R_2TR_2T$	3	1.594	1.240	1 $ii\ R_1R_1\ R_2TR_2T$	0	.797	.797
2 $Ii\ R_1R_1\ R_2TR_2T$	1	1.594	.221	2 $ii\ R_1R_1\ R_2Tr_2t$	1	1.594	.221
4 $II\ R_1r_1\ R_2Tr_2t$	5	3.188	1.030	2 $ii\ R_1r_1\ R_2TR_2T$	2	1.594	.104
4 $Ii\ R_1R_1\ R_2Tr_2t$	2	3.188	.443	4 $ii\ R_1r_1\ R_2Tr_2t$	3	3.188	.011
4 $Ii\ R_1r_1\ R_2TR_2T$	2	3.188	.443	1 $II\ r_1r_1\ r_2tr_2t$	1	.797	.051
8 $Ii\ R_1r_1\ R_2Tr_2t$	4	6.376	.885	2 $Ii\ r_1r_1\ r_2tr_2t$	1	1.594	.221
1 $II\ R_1R_1\ r_2tr_2t$	1	.797	.051	1 $ii\ R_1R_1\ r_2tr_2t$	3	.797	6.089
2 $II\ R_1r_1\ r_2tr_2t$	0	1.594	1.594	2 $ii\ R_1r_1\ r_2tr_2t$	1	1.594	.221
2 $Ii\ R_1R_1\ r_2tr_2t$	1	1.594	.221	1 $ii\ r_1r_1\ R_2TR_2T$	2	.797	1.816
4 $Ii\ R_1r_1\ r_2tr_2t$	3	3.188	.011	2 $ii\ r_1r_1\ R_2Tr_2t$	1	1.594	.221
1 $II\ r_1r_1\ R_2TR_2T$	1	.797	.051	1 $ii\ r_1r_1\ r_2tr_2t$	1	.797	.051
2 $II\ r_1r_1\ R_2Tr_2t$	3	1.594	1.240				

$P=0.506$. $\chi^2=25.227$.

Table 10 shows the assumed genotypes for the F_2 plants, together with the observed and expected numbers of each when grouped according to their breeding behavior. The data conform closely to the theoretical, although three of the expected genotypes were not obtained. However, with 51 individuals and 27 different classes, the distribution is considered a very good fit. The values of χ^2 and P are 25.227 and 0.506, respectively.

In order to submit the data to a more critical test, 56 F_3 plants from F_2 plant No. 12 (Table 9) which segregated like the F_1 were tested in the greenhouse during the winter. Data on the breeding

behavior of this family of plants should be equivalent to the data on the breeding behavior of the F_2 plants. Adding these 56 F_3 plants to the 51 F_2 plants tested makes a total of 107 plants which were submitted to the progeny test. With the 107 plants all the assumed genotypes were obtained, though from the 56 F_3 plants 3 expected genotypes were not produced. These 3, however, were different from the 3 not obtained in the F_2 generation. The value of χ^2 for the 107 plants was 27.305 and for P, 0.394. The P value here was no larger than for the F_2 distribution. The probable errors and P values were determined for the total of the F_3 plants in each class of the 27 genotypes which segregated. By referring to Table 9 it will be noted that for all plants heterozygous for one factor the deviations from a 3:1 ratio when compared with their probable errors are in no case too large to be considered as due to other influences than random sampling. Also, for all plants heterozygous for two factors the P values although not high indicate good fits for a 9:3:3:1 ratio. On the other hand the P value (0.026) for plants segregating for three factors is low, indicating a poor fit. Just why the P value here is so low as compared with the P value for the same distribution in the F_2 can not be explained satisfactorily. It will be observed, however, that the yellow-brown class is the one that deviates most. Turning to Table 8 for the F_2 distribution, it may be noted that the number of plants in this class, although a little high, is not excessive. In looking over data presented by Owen (*6*) on similar crosses the number of plants in this class does not seem to be excessive. It is thought, therefore, that the deviation here is due to chance.

CROSSES INVOLVING EYEBROW-COLOR PATTERN

The inheritance of eyebrow-color pattern has been reported by Nagai and Saito (*2*) and by Owen (*4, 5*). Nagai and Saito reported this color pattern to be due to a restriction factor, K, which inhibits or restricts the black color from developing uniformly over the seed coat. Owen changed the nomenclature slightly. The restriction or inhibiting factor was retained but put into his multiple allelomorphic, I, series and called I^k. This I^k factor was reported to be dominant to the recessive i factor which gives self-black or brown colors, but recessive to I^h and I^i, factors for yellow.

The writer has been able to obtain two different crosses which involve the eyebrow-pattern factor. In cross No. 83 (Ogemaw × Black Eyebrow) the Ogemaw is a self-colored reddish-brown variety. The Black Eyebrow has a brown ground color with a saddle of black over the sides. The F_1 plants bore seeds like the Black Eyebrow parent. In the F_2 four different color types were observed—the black eyebrow, brown eyebrow, self-black, and self-brown. With these four different color types it is evident that at least two factors are involved. A total of 74 F_2 plants was obtained. For a dihybrid ratio with 74 individuals the numbers expected are 41.6:13.9:13.9:4.6. The observed ratio was 39:11:20:4. The χ^2 value for this distribution is 3.55 and the P value 0.32.

In the second cross, No. 183 (Ito San × Black Eyebrow), involving the eyebrow-pattern color, two F_1 plants were obtained which were given the numbers 183–1 and 183–2. The Ito San parent had a yellow seed coat, a light, brown hilum, and was mottled to a considerable

extent. The Black Eyebrow parent has already been described above. The F_1 plants from these crosses were yellow mottled with black. In the F_2 generation four color types were observed—yellow mottled with black, yellow mottled with brown, black eyebrow pattern, and brown eyebrow pattern. It is evident that at least two factors are involved in this cross and that one of the two is the same as in cross No. 83 and the other is different. Considering the F_2 data from the two F_1 plants (183–1 and 183–2) together there were 150 F_2 plants obtained. For this number of plants, with two pairs of factors involved, the expected ratio is 84.4:28.1:28.1:9.4. The actual ratio was 82:28:25:15. With the exception of the last class represented by the brown eyebrow pattern type, which is a little high, the numbers conform well to the expected. The values for χ^2 and P in this cross are 3.795 and 0.288, respectively. The F_2 data for these crosses are presented in Table 11.

TABLE 11.—*F^2 Progenies from crosses between varieties of soybeans which involved the eyebrow-pattern factor*

Cross	Mottled		Eyebrow pattern		Self-color	
	Yellow black	Yellow brown	Black	Brown	Black	Brown
Ogemaw × Black Eyebrow:						
No. 83–1			23	6	8	2
No. 83–2			16	5	12	2
Total observed			39	11	20	4
Expected [a]			41.6	13.9	13.9	4.6
Ito San×Black Eyebrow:						
No 183–1	43	10	11	9		
No 183–2	39	18	14	6		
Total observed	82	28	25	15		
Expected [b]	84.4	28.1	28.1	9.4		

[a] $\chi^2=3.6$. $P=0.3$.

[b] $\chi^2=3.8$. $P=0.3$.

The factor I^k suggested by Owen (*5*) is used to designate the restriction gene in the eyebrow-pattern color, and since the Black Eyebrow variety contains black pigment it must also contain R_1 and R_2. The Ogemaw variety is self-colored and must contain i. It has already been shown that it contains a factor $r_1{}^o$ which is recessive to r_1, and it will be shown later that this factor $r_1{}^o$ is also recessive to R_1. Assuming then that the Black Eyebrow variety is of the composition $I^k R_1$ and the Ogemaw variety $ir_1{}^o$, the data presented for cross No. 83 can be explained.

In cross No. 183 it has already been shown that the brown hilum of the Ito San variety is due to a pigment factor r_1, which is recessive to R_1. Assuming that I restricts the brown pigment to the region of the hilum, the composition of the Ito San variety becomes Ir_1. By crossing this variety with the variety Black Eyebrow, the phenotypes shown in Table 11 for this cross would be expected in approximately a 9 : 3 : 3 : 1 ratio, which fits the facts observed.

In order to test these crosses further, F_3 plants were grown from 70 of the F_2 plants in cross No. 83 and from 148 F_2 plants in cross No. 183. The complete data for the F_3 generation in these crosses are given in Tables 14 and 15. Tables 12 and 13 show the assumed genotypes for the F_2 plants, together with the observed and expected numbers of each when grouped according to their breeding behavior.

TABLE 12.—*Genotypic distribution of F_2 plants as shown by their breeding behavior in the F_3 generation*

[Cross No. 83, Ogemaw (*ii* $r_1{}^o r_1{}^o$ R_2TR_2T)× Black Eyebrow (I^kI^k R_1R_1 R_2TR_2T)]

Genotype F_2 plant	Observed	Expected	$\frac{(o-c)^2}{c}$	Genotype F_2 plant	Observed	Expected	$\frac{(o-c)^2}{c}$
1 R_1R_1 I^kI^k	4	4.38	0.032	2 R_1r_1 *ii*	17	8.76	7.751
2 R_1R_1 I^ki	6	8.76	.870	1 r_1r_1 I^kI^k	4	4.38	.032
2 R_1r_1 I^kI^k	9	8.76	.007	2 r_1r_1 I^ki	7	8.76	.354
4 R_1r_1 I^ki	17	17.52	.015	1 r_1r_1 *ii*	3	4.38	.434
1 R_1R_1 *ii*	3	4.38	.434				

P=0.271. χ^2=9.929.

TABLE 13.—*Genotypic distribution of F_2 plants as shown by their breeding behavior in the F_3 generation*

[Cross 183, Ito San (*II* r_1r_1 R_2TR_2T)×Black Eyebrow (I^kI^k R_1R_1 R_2TR_2T)]

Genotype F_2 plant	Observed	Expected	$\frac{(o-c)^2}{c}$	Genotype F_2 plant	Observed	Expected	$\frac{(o-c)^2}{c}$
1 *II* R_1R_1	4	9.25	2.979	2 II^k r_1r_1	18	18.50	0.014
2 *II* R_1r_1	18	18.50	.014	1 I^kI^k R_1R_1	12	9.25	.817
2 II^k R_1R_1	20	18.50	.122	2 I^kI^k R_1r_1	13	18.50	1.635
4 II^k R_1r_1	39	37.00	.108	1 I^kI^k r_1r_1	15	9.25	3.574
1 *II* r_1r_1	9	9.25	.006				

P=0.314. χ^2=9.269.

In Table 12, for cross No. 83, it may be seen that the class heterozygous for self-black contains an excess of the F_2 genotypes. When these data are compared with data in Table 11 it is noticed that the number of F_2 plants with black is also larger than expected. Since all but two plants in this group were tested, an excess of the segregating self-black plants would be expected. In Table 13, for cross No. 183, it may be observed that the genotypic ratio for the F_2 plants approaches very closely to the expected. The values of χ^2 and P for the F_2 genotypic distribution are 9.93 and 0.27 for cross No. 83 and 9.27 and 0.31 for cross No. 183.

TABLE 14.—*Assumed genotypes together with breeding behavior of F_2 plants tested in the F_3 generation*

[Cross No. 83 Ogemaw (*ii* $r_1{}^o r_1{}^o$ R_2R_2)×Black Eyebrow (I^kI^k R_1R_1 R_2R_2)]

Genotype and F_2 plant No.	Phenotypes observed in the F_3 generation				Deviation P. E.
	Eyebrow pattern		Self-colored		
	Black	Brown	Black	Brown	
I^kI^k R_1R_1 R_2R_2:					
1-30	12				
1-34	36				
2-2	83				
2-12	44				
I^kI^k $r_1{}^o r_1{}^o$ R_2R_2:					
1-7		37			
1-20		60			
1-26		84			
2-26		44			
ii R_1R_1 R_2R_2:					
1-11			27		
1-23			56		
2-5			11		
ii $r_1{}^o r_1{}^o$ R_2R_2:					
1-5				80	
2-7				61	
2-21				43	

TABLE 14.—*Assumed genotypes together with breeding behavior of F_2 plants tested in the F_3 generation*—Continued

[Cross No. 83 Ogemaw ($ii\ r_1{}^o r_1{}^o\ R_2R_2$)×Black Eyebrow ($I^kI^k\ R_1R_1\ R_2R_2$)]

Genotype and F_2 plant No.	Phenotypes observed in the F_3 generation				Deviation P. E.
	Eyebrow pattern		Self-colored		
	Black	Brown	Black	Brown	
$I^kI^k\ R_1r_1{}^o\ R_2R_2$:					
1-1	34	11			
1-8	14	8			
1-9	19	12			
1-17	46	19			
1-24	30	16			
1-29	43	14			
1-32	7	1			
1-35	7	7			
2-34	26	7			
Total	226	95			2.82
$I^ki\ R_1R_1\ R_2R_2$:					
1-21	31		10		
1-36	13		5		
2-24	37		10		
2-32	4		4		
2-35	52		14		
2-31	22		4		
Total	159		47		1.07
$I^ki\ r_1{}^or_1{}^o\ R_2R_2$:					
1-10		15		7	
1-18		6		1	
1-33		33		11	
2-6		16		4	
2-8		96		44	
2-23		19		7	
2-28		49		15	
Total		234		89	1.57
$R_1r_1{}^o\ R_2R_2$:					
1-12			24	4	
1-13			16	7	
1-14			38	11	
1-25			80	32	
1-27			61	22	
1-37			28	20	
2-3			53	20	
2-9			14	2	
2-11			1	2	
2-13			30	10	
2-14			36	17	
2-15			31	12	
2-16			12	3	
2-19			48	13	
2-20			51	22	
2-22			50	17	
2-36			33	12	
Total			606	225	2.14
$I^ki\ R_1r_1{}^o\ R_2R_2$:					
1-2	10	2	3	1	
1-3	1	1	2	1	
1-4	21	3	6	2	
1-6	13	3	5	2	
1-15	14	5	4	3	
1-19	21	10	8	7	
1-31	25	9	9	4	
1-38	27	6	8	3	
1-40	42	13	17	2	
2-4	40	16	11	5	
2-10	28	12	9	4	
2-18	38	16	12	5	
2-25	13	4	4	1	
2-27	9	5	2	1	
2-30	31	8	14	4	
2-33	36	8	11	2	
2-37	25	4	14	4	
Total	395	125	139	51	

χ^2=1.79. P=0.62.

TABLE 15.—*Assumed genotypes, together with breeding behavior of F_2 plants tested in the F_3 generation*

[Cross No. 183 Ito San ($II\ r_1r_1\ R_1R_2$)×Black Eyebrow ($I^kI^k\ R_1R_1\ R_2R_2$)]

Genotypes and F_2 plant No.	Phenotypes observed in the F_3 generation				Deviation P. E.
	Mottled		Eyebrow pattern		
	Yellow black	Yellow brown	Black	Brown	
$II\ R_1R_1\ R_2R_2$:					
1-45	57				
2-10	138				
2-29	63				
2-58	58				
$II\ r_1r_1\ R_2R_2$:					
1-28		89			
1-46		28			
1-68		63			
2-17		70			
2-23		64			
2-27		71			
2-30		63			
2-43		68			
2-72		66			
$I^kI^k\ R_1R_1\ R_2R_2$:					
1-3			61		
1-12			48		
1-31			104		
1-40			49		
1-71			64		
1-73			65		
2-25			61		
2-28			68		
2-42			58		
2-44			65		
2-49			49		
2-75			59		
$I^kI^k\ r_1r_1\ R_2R_2$:					
1-2				91	
1-10				66	
1-18				11	
1-33				76	
1-35				35	
1-38				55	
1-47				63	
1-59				80	
1-63				66	
2-9				55	
2-13				54	
2-19				56	
2-21				31	
2-24				27	
2-65				29	
$II\ R_1r_1\ R_2R_2$:					
1-1	69	25			
1-6	42	10			
1-8	73	21			
1-20	61	26			
1-22	96	32			
1-29	42	13			
1-41	41	13			
1-52	45	13			
1-53	32	13			
2-15	45	12			
2-26	45	15			
2-39	76	30			
2-41	48	10			
2-47	111	41			
2-51	117	37			
2-63	69	21			
2-67	74	24			
2-73	61	15			
Total	1,147	371			0.75

TABLE 15.—*Assumed genotypes, together with breeding behavior of F_2 plants tested in the F_3 generation*—Continued

[Cross No. 183 I to 8an ($II\ r_1r_1\ R_2R_2$)×Black Eyebrow ($I^bI^b\ R_1R_1\ R_2R_2$)]

Genotypes and F_2 plant No.	Phenotypes observed in the F_2 generation				Deviation / P. E.
	Mottled		Eyebrow pattern		
	Yellow black	Yellow brown	Black	Brown	
$II^b\ R_1R_1\ R_2R_2$:					
1-11	59		20		
1-15	59		30		
1-17	103		38		
1-19	80		37		
1-27	77		20		
1-30	37		16		
1-32	77		37		
1-51	78		23		
1-54	81		26		
1-61	106		31		
1-66	72		31		
1-69	48		10		
2-4	89		28		
2-5	46		12		
2-8	105		30		
2-45	22		10		
2-48	44		18		
2-50	72		27		
2-69	83		21		
2-74	29		14		
Total	1,367		479		1.39
$II^b\ r_1r_1\ R_2R_2$:					
1-5		113		33	
1-44		74		28	
1-49		119		35	
1-55		74		16	
1-60		82		22	
1-64		97		33	
1-72		99		35	
2-2		119		35	
2-6		53		17	
2-16		22		9	
2-18		76		32	
2-20		99		45	
2-32		55		14	
2-37		92		28	
2-52		67		13	
2-55		25		4	
2-59		111		37	
2-60		111		29	
Total		1,488		465	1.80
$I^bI^b\ R_1r_1\ R_2R_2$:					
1-21			64	18	
1-23			91	38	
1-25			26	9	
1 50			50	17	
1-58			81	18	
2-22			95	32	
2-33			75	26	
2-46			19	7	
2 61			64	23	
2-62			99	44	
2-64			36	13	
2-76			104	22	
2-77			45	10	
Total			849	277	.46
$II^bR_1r_1\ R_2R_2$:					
1-4	48	16	19	6	
1-7	33	11	11	4	
1-9	13	7	4	2	
1-13	57	15	16	8	
1-14	87	29	20	12	
1-16	57	26	18	6	
1-24	43	10	14	4	

TABLE 15.—*Assumed genotypes, together with breeding behavior of F_2 plants tested in the F_3 generation*—Continued

[Cross No. 183 I to San ($II\ r_1r_1\ R_2R_2$)×Black Eyebrow ($I^hI^h\ R_1R_1\ R_2R_2$)]

Genotypes and F_2 plant No.	Phenotypes observed in the F_3 generation				Deviation / P. E.
	Mottled		Eyebrow pattern		
	Yellow black	Yellow brown	Black	Brown	
$II^h\ R_1r_1\ R_2R_2$—Continued.					
1-26	51	20	16	7	
1-34	25	11	9	7	
1-36	22	9	19	4	
1-37	47	10	10	6	
1-39	25	6	12	3	
1-42	67	20	20	6	
1-43	79	17	15	7	
1-48	38	28	18	4	
1-56	70	16	22	9	
1-57	36	12	12	3	
1-62	77	15	24	12	
1-65	97	17	28	9	
1-67	34	7	8	2	
1-70	62	22	15	11	
2-1	19	7	7	0	
2-3	99	40	40	15	
2-7	58	17	16	3	
2-11	41	19	12	4	
2-14	43	13	15	5	
2-31	33	22	12	1	
2-34	35	13	10	3	
2-35	43	9	13	3	
2-38	94	22	14	9	
2-40	111	24	24	10	
2-53	34	8	9	1	
2-54	91	22	30	9	
2-56	58	19	17	10	
2-57	94	32	20	12	
2-58	49	11	21	7	
2-66	45	15	13	6	
2-68	55	16	15	11	
2-71	71	23	23	8	
Total	2,144	656	641	249	

χ^2=9.3. P=0.03.

By referring to Table 14 for cross No. 83 and Table 15 for cross No. 183, it may be seen that the deviation from a 3:1 ratio for each of the classes segregating for one pair of factors when compared with its probable error is not too large for a good fit. Tables 14 and 15 also contain data for F_2 plants of the above crosses segregating as the F_1. The values of χ^2 and P for cross No. 83 (Table 14) are 1.79 and 0.62, respectively, and for cross No. 183 (Table 15) are 9.32 and 0.03, respectively. In the last case the fit is poor, but since the rest of the F_3 data and the results obtained for the F_2 generation conform well to the expected, it is thought that the inheritance can well be explained by assuming two independent factors.

SELECTION INVOLVING YELLOW AND BROWN SEED-COAT COLORS

Owen (*5*) has reported results of crosses between varieties of soybeans with yellow seed coats and varieties with brown seed coats. In his work approximately three plants bearing yellow seeds to one with self-colored seeds were obtained. Two restriction factors for pigment I^h or I^i and their recessive allelomorph were assumed in order to explain his findings. When a variety having a yellow seed coat and

black hilum was crossed with a brown-seeded variety, F_2 plants bearing self-colored black seeds and plants bearing self-colored brown seeds were obtained. When a variety having a yellow seed coat and a brown hilum was crossed with a variety having a brown seed coat only F_2 plants with yellow seeds and brown hilums or self-brown seeds were obtained.

During these investigations a probable natural hybrid, selection No. 31, was found to be segregating for yellow and brown seed-coat color. The original plant, selection No. 31, bore yellow mottled brown seeds and had tawny pubescence. From the seeds of this plant 43 progenies (the F_2 plants) were grown. Of these, 28 bore yellow seeds which were mottled with brown and 15 seeds which were self-brown. All the F_2 plants had tawny pubescence. For a 3:1 ratio one would expect from 43 plants, 32.25 to bear yellow seeds mottled with brown and 10.75 to bear self-brown seeds. The deviation 4.25 ± 1.92 is considered a fairly close fit, and the original plant is thought to have been hybrid for a single pair of factors. Using *I* as the restriction factor for pigment color, and its recessive allelomorph *i* to designate self-color, the plant must have had the genetic constitution *Ii*. A natural cross between the Ito San variety and the Soysota variety would give such a genotype.

ALLELOMORPHIC SERIES OF FACTORS WHICH RESTRICT BLACK AND BROWN PIGMENTS

Owen (*5*) has reported a series of allelomorphs I^h, I^t, I^k, and *i* which have certain inhibiting effects upon the development of pigment colors in the seed coat. Data taken from certain crosses obtained in the present studies show rather conclusively that such a series does exist. However, the writer has not been able to differentiate between I^h and I^t. Owen also had some trouble in making this classification, especially where mottling took place to any extent. All progenies from crosses used in the present studies where yellow color was involved mottled badly. Possibly this is the reason that I^h and I^t could not be distinguished. Since the author has not been able to differentiate between I^h and I^t, any plants with yellow seed coat regardless of the color of the mottling or color of hilum have been designated as *I*. The series used in this paper is *I*, I^k, and *i*.

The evidence for such a series is obtained from F_2 data on the following crosses:

Ito San (*II*) × Black Eyebrow (I^kI^k)—3 plants with yellow-mottled seeds to 1 with the eyebrow pattern.

Black Eyebrow (I^kI^k) × Ogemaw (*ii*)—3 plants with the eyebrow pattern to 1 with self-colored seeds.

Manchu (*II*) × Wisconsin Black (*ii*)—3 plants with yellow-mottled seeds to 1 with self-black.

The breeding behavior observed in the above crosses is good evidence that the three factors *I*, I^k, and *i* are allelomorphic. No other explanation would likely give only the two types which go into the cross in the F_2 generation. Data for the first two combinations are shown in Table 11 and for the last combination in Table 8. These crosses lend support to Owen's hypothesis that a multiple allelomorphic series exists at the *I* locus by bringing in data from one combination, Black Eyebrow (I^kI^k) × Ogemaw (*ii*), which Owen did not obtain.

ALLELOMORPHIC SERIES OF FACTORS WHICH INFLUENCE THE INTENSITY OF BLACK AND BROWN PIGMENTS

Owen (*5*) has postulated that an allelomorphic series which influences the pigment color is situated near the *t* locus for pubescence color. In these studies no evidence was obtained which indicated an allelomorphic series at this locus. There is, however, considerable indication that such a series exists at the R_1 locus. Evidence for such a series was obtained from F_2 data from the following crosses:

Manchu (R_1R_1) × Ito San (r_1r_1)—3 plants bearing seeds with black pigment to 1 with light brown.

Manchu (R_1R_1) × Wisconsin Black (R_1R_1)—all plants bearing seeds with black pigment.

Wisconsin Black (R_1R_1) × Soysota (r_1r_1)—3 plants bearing seeds with black pigment to 1 with light brown.

Soysota (r_1r_1) × Ogemaw ($r_1{}^or_1{}^o$)—3 plants bearing seeds with light-brown pigment to 1 with reddish brown.

Black Eyebrow (R_1R_1) × Ogemaw ($r_1{}^or_1{}^o$)—3 plants bearing seeds with black pigment to 1 with reddish brown.

Black Eyebrow (R_1R_1) × Ito San (r_1r_1)—3 plants bearing seeds with black pigment to 1 with light brown.

Data for the first combination may be obtained by referring to Table 1; for the second combination, Table 8; for the third, paragraph involving black and brown seed coat, on page 835; for the fourth, Table 2; and for the fifth and sixth, Table 11.

With the above combinations behaving as they do it seems advisable to assume that an allelomorphic series exists at the R_1 locus. No other explanation would likely give only the types which go into the crosses in the F_2 generation. Two questions which might arise are: (1) Is Black Eyebrow really of the constitution R_1R_1, and, (2), what would happen should Wisconsin Black be crossed with Black Eyebrow? In the discussion of cross No. 83, it was shown that four different color types appear in the F_2 generation—the black eyebrow, reddish-brown eyebrow, self-black, and self-reddish brown. Without R_1 in the Black Eyebrow variety no self-blacks could come out of this cross because it is crossed with a variety Ogemaw which contains no black pigment. No cross has been made between Wisconsin Black and Black Eyebrow, but from the same cross, No. 83, 6 plants of the Black Eyebrow type segregated for approximately 3 plants of the Black Eyebrow type to 1 self-black. From this cross 17 self-black F_2 plants of the genetic constitution $R_1r_1{}^o$ were obtained which gave a good 3:1 ratio of self-blacks and reddish browns. The data for these combinations may be seen by referring to Table 14.

DISCUSSION

As previously stated, a number of workers, Nagai (*1*), Nagai and Saito (*2*), Woodworth (*7*), and Owen (*4*, *5*) have studied these pigment colors genetically. Certain similarities have been observed in their findings which may be mentioned.

Nagai (*1*) reported studies of seed-coat inheritance from crosses involving the pigments discussed in this paper. According to his hypothesis two complementary factors, *C* and *R*, were responsible for the formation of black pigment. With *CR* the color was black, with *cR* the color was imperfect black, with *Cr* the color was brown, and with both recessives, *cr*, the color was buff.

Nagai and Saito (*2*) reported three factors, *H*, *I*, and *K*, which inhibit the black or brown pigment. With *H* present no black or brown pigment develops, and the seed coat is yellow or green. The factor *I* inhibits or restricts the pigment to the hilum, and *K* restricts the pigment to give the eyebrow pattern.

Woodworth (*7*) in a study of black and brown pigments in the hilum observed two complementary factors for the inheritance of black and brown pigments. These two color factors were designated *H* and *B*.

Owen (*4*, *5*) in an extensive study of soybean inheritance observed two factors, R_1 and R_2, for black pigments. He also reported an allelomorphic series of factors which restrict the pigments entirely or to certain areas on the seed coat.

It can not be definitely determined whether the factors reported by the various workers are the same. There are, however, many resemblances between certain of the factors which will be brought out later in the discussion.

As stated earlier, the work of Owen is more extensive than that of previous investigators. Therefore to avoid confusion in nomenclature it seems preferable to use his symbols as far as possible in explaining data collected in these studies. Before advancing further in the discussion the assumed genotypes used for all of the different crosses should be presented. These are as follows:

Wisconsin Black ($ii\ R_1R_1\ R_2TR_2T$) × Mandarin ($II\ r_1r_1\ r_2{}^lr_2{}^l$).
Ogemaw ($ii\ r_1{}^or_1{}^o\ R_2TR_2T$) × Soysota ($ii\ r_1r_1\ R_2TR_2T$).
Black Eyebrow ($I^kI^k\ R_1R_1\ R_2TR_2T$) × Ogemaw ($ii\ r_1{}^or_1{}^o R_2TR_2T$).
Manchu ($IIR_1R_1\ R_2TR_2T$) × Ito San ($II\ r_1r_1\ R_2TR_2T$).
Ito San ($II\ r_1r_1\ R_2TR_2T$) × Black Eyebrow ($I^kI^k\ R_1R_1\ R_2TR_2T$).
Manchu ($II\ R_1R_1\ R_2TR_2T$) × Wisconsin Black ($ii\ R_1R_1\ R_2TR_2T$).
Manchu ($II\ R_1R_1\ R_2TR_2T$) × Wilson ($ii\ R_1R_1\ R_2TR_2T$).
Wisconsin Black ($ii\ R_1R_1\ R_2TR_2T$) × Soysota ($ii\ r_1r_1\ R_2TR_2T$).
Natural hybrid No. 10 ($II\ r_1r_1\ R_2TR_2T$) × ($II\ r_1r_1\ r_2{}^lr_2{}^l$).[5]
Natural hybrid No. 31 ($ii\ r_1r_1\ R_2TR_2T$) × ($II\ r_1r_1\ R_2TR_2T$).[5]

In the above hypothesis it is assumed that there is an allelomorphic series at the *I* locus. Gene *I* is a restriction factor which must be present in all varieties with yellow seed coats. I^k is an allelomorph to *I* which restricts black or brown color so as to give the eyebrow-color pattern. Gene *i* is a recessive allelomorph to both *I* and I^k and must be present in order to get self-colors of any kind. A second series of allelomorphs (R_1, r_1, and $r_1{}^o$) is assumed at the R_1 locus. The factor R_1 causes the development of anthocyanin pigment. It is also complementary to R_2, giving the intense black color. With R_1r_2 the color is imperfect black. Gene r_1 is recessive to R_1, and in the presence of R_2 gives a light brown color. With r_1r_2 the color is buff. The factor $r_1{}^o$ is recessive to either R_1 or r_1. When present with R_2 it gives a rich reddish-brown pigment. No crosses involving r_2 and $r_1{}^o$ have been obtained. R_2 is closely lined with *T*, a factor for tawny pubescence, and no crossing over has been observed.

Although the symbols used by Owen (*5*) have been followed as closely as possible it has been necessary to make some departures in order to explain satisfactorily data gathered during this study. The first change which will be discussed is the difference in the *I* series of

[5] The last two combinations were natural hybrids and the parents are unknown. The genetic composition has been assumed from their breeding behavior.

allelomorphs. In his hypothesis Owen has used two factors in this series I^h and I^i which the writer has been unable to use. Quoting from Owen (*5*, *p. 56*):

> I^h under the proper environmental conditions inhibits all pigment formation in the seed coat; I^i inhibits pigments to the hilum. * * * It should be clearly kept in mind that environmental conditions unfavorable for pigment formation are necessary in order for these restriction factors to fully express themselves.

Nagai and Saito (*2*) have also described a factor *H* which acts very much like the I^h factor described by Owen.

As already shown, the present writer has been unable to make such a classification. Almost all plants with yellow seed coats used in these studies have been mottled to a considerable extent. Occasionally plants occurred which were reasonably free from mottling. Others appeared which were extremely mottled, but when tested in the F_3 there was no indication of these differences being genetic. It has also been noticed that there was just about as much variation in mottling among seeds on a plant as there was among the different plants. During the course of this study there has been no occasion for distinguishing between I^h and I^i. Therefore, no attempt has been made to separate the two factors. *I* alone has been used to designate the restriction of black and brown pigments giving the yellow or yellow-mottled color. It is quite possible that plants used for these crosses were genetically different from those used by Owen. Another possibility is that environmental conditions at Ames, Iowa, were such that the color pigments were brought out much more distinctly than they were at Madison, Wis. It has been shown by Hollowell[6] and Owen (*3*) that different environmental conditions do play an important part in the degree of mottling. This may account for different degrees of mottling which the author has often noticed on the same plant and which has been reported by Woodworth and Cole (*9*). Hollowell,[7] and Owen (*3*). Another point which might be mentioned is that all plants used in these studies were spaced 4 to 6 inches apart in the rows and grown on land which was fairly fertile. Both spacing and soil fertility have been found by Owen and Hollowell to influence the degree of mottling.

Some changes have been made in the uses of the two *R* factors for black and brown pigments. In these studies the author has found it necessary to postulate a series of allelomorphs at the locus of R_1, but it has not been necessary to use the allelomorphic series at the locus of R_2, as postulated by Owen (*5*). The factor R_1—which is probably the *R* factor reported by Nagai (*1*), the *B* factor reported by Woodworth (*7*), and the R_1 factor reported by Owen (*5*)—causes the development of anthocyanin pigment. However, it does not, when present alone, give the intense-black color which we are accustomed to see in black varieties of soybeans. Only when R_1 appears in the zygote with R_2 is the intense black color produced. In combination with r_2, R_1 causes anthocyanin pigment to develop, but it gives a dilute black instead of the ordinary black color. The factor R_2 is probably the same as the chromongen factor *C* described by Nagai (*1*), the *H* factor described by Woodworth (*7*), and the R_2 factor described by Owen (*5*). The factor R_2, although described by Owen as being a

[6] HOLLOWELL, E. A. FACTORS INFLUENCING THE MOTTLING OF THE SOYBEAN SEED COAT. 1924. Unpublished master's thesis. Copy on file, library, Iowa State College, Ames.

[7] HOLLOWELL, E. A. Op. cit.

factor for black pigment, does not cause the development of anthocyanin pigment alone, but acts in a complementary way with R_1 to produce black. This fact has also been observed by Nagai and Saito (*2*) and Woodworth (*7*). When R_2 is present in the zygote with r_1, a light-brown color is produced. With both r_1 and r_2 the color is buff.

As R_1 and R_2 are used in this paper, data from all crosses involving black and brown pigment colors in the seed coat thus far reported can be explained. However, in one cross, No. 142 (Selection No. 3 × Selection No. C 3) reported by Owen, a slight modification must be made. In this cross flower color is also involved, and in order to explain the data presented by Owen with the hypothesis here presented it is necessary to assume that W, the factor for purple-flower color, is also complementary to R_1 to give the imperfect-black color in the seed coat. In using the hypothesis presented by Owen the W factor is considered as complementary to r_2 instead of R_1. This assumption brings in certain complications which are not easily explained. Since the main difference in the use of R_1 and R_2 centers around this one cross, it is thought advisable to discuss the cross from the standpoint of the two hypotheses. According to Owen's hypothesis, Selection No. 3 possessed the following constitution: *ii* r_1r_1 R_2TR_2T *WW* and Selection No. C 3, I^hI^h r_1r_1 r_2tr_2t *ww*. For this combination the F_2 phenotypes would be as follows:

27	I^h r_1 R_2TW	Yellow, mottled with black—tawny pubescence—purple flower.
9	I^h r_1 R_2Tw	Yellow, mottled with black—tawny pubescence—white flower.
9	I^h r_1 r_2tW	Yellow, mottled with imperfect black—Gray pubescence—purple flower.
3	I^h r_1 r_2tw	Yellow, mottled with buff—gray pubescence—white flower.
9	i r_1 R_2TW	Self-black—tawny pubescence—purple flower.
3	i r_1 R_2Tw	Self-black—tawny pubescence—white flower.
3	i r_1 r_2tW	Self-imperfect black—gray pubescence—purple flower.
1	i r_1 r_2tw	Self-buff—gray pubescence—white flower.

The data presented by Owen are explained very satisfactorily by the above hypothesis, but when one considers the genetic relationship of the two varieties used in this cross along with other crosses which are similar, certain conditions arise which can not well be explained.

By observing the phenotypes it will be noticed that I^h r_1 r_2t W has been used to designate yellow mottled with imperfect black; but this is the same formula which has to be used for the Mandarin variety. The Mandarin variety has a yellow seed coat. It, then, must contain I^h, the restriction factor.[8] It has buff pigment in the hilum and is represented by Owen as well as by the present writer as containing r_1. It has gray pubescence; therefore it must contain r_2 because r_2 is completely linked with t, factor for gray pubescence. Finally, the Mandarin variety has purple flowers and therefore must contain W. Accordingly, the formula I^h r_1 r_2t W in the Mandarin variety means yellow mottled with buff. Here it is assumed to mean yellow mottled with imperfect black. A second inconsistency in the hypothesis is found in the phenotype i r_1R_2T W, which here is used to designate self-black. But this is the same formula which has to be used in the Soysota variety with a brown seed coat. Soysota is self-colored and

[8] According to the hypothesis used by the present author I^h should be I.

therefore it must have *i*. It has been designated by Owen as well as by the writer as having r_1, and it must contain R_2TW because it has tawny pubescence and purple flowers. A third inconsistency in Owen's hypothesis is found in the phenotype *i* r_1 r_2t *W*, which here is used to designate self-colored imperfect black, but in certain crosses reported by Owen this formula has been used to designate plants which bore buff-colored seeds and purple flowers. There is still one other feature of the hypothesis which seems to be rather unsatisfactory. This will be observed in the phenotype I^h r_1 R_2T *W*, which here is used to designate yellow mottled with black. In Ito San, a yellow-seeded variety with a brown hilum which often mottles brown, I^h and r_1 have been reported by Owen to be present. The studies made by the present author bear out this assumption. But the Ito San variety has purple flowers and tawny pubescence. It therefore should have the genetic constitution I^h r_1 R_2TW. Owen, too, has noticed this fact, and in order to explain his data when the Ito San variety is crossed with Selection No. 3 (the black parent) of the above cross, he assumed an allelomorphic series to exist at the R_2 locus, giving the Ito San variety the formula I^h r_1r_2' *TW*.

These rather inconsistent uses of formulae seem to be unnecessary, and the author is presenting a hypothesis for the cross which he believes answers all requirements in this cross and at the same time is in agreement with the factorial composition of other crosses used by previous workers as well as in the present studies.

The genetic constitution of Owen's Selection No. 3 (black parent) is assumed to be *ii* R_1R_1 R_2TR_2T *WW* and for Selection No. C 3 (yellow parent), *II* R_1R_1 r_2tr_2t *ww*. The F_2 phenotypic ratio is as follows:

27	*I* R_1 R_2T *W*------	Yellow, mottled with black—tawny pubescence—purple flower.
9	*I* R_1 R_2T *w*------	Yellow, mottled with black—tawny pubescence—white flower.
9	*I* R_1 r_2t *W*-------	Yellow, mottled with imperfect black—gray pubescence—purple flower.
3	*I* R_1 r_2t *w*--------	Yellow, mottled with buff—gray pubescence—white flower.
9	*i* R_1 R_2T *W*------	Self-black—tawny pubescence—purple flower.
3	*i* R_1 R_2T *w*-------	Self-black—tawny pubescence—white flower.
3	*i* R_1 r_2t *W*-------	Self-imperfect black—gray pubescence—purple flower.
1	*i* R_1 r_2t *w*--------	Self-buff—gray pubescence—white flower.

With the above hypothesis it is necessary to assume that the factor *W* for purple flower color is complementary to R_1, giving the imperfect-black color in the seed coat. With R_1 and *w* the color of the pigment is buff. It may be said that the imperfect black shows considerable buff pigment and that a slight diluting of the imperfect black by *w* would produce a buff color. This diluting effect is in accord with other observations. Woodworth (*8*) has noticed that the purple flower color was closely associated with purple hypocotyl and that with white flowers no purple pigment was developed. This point, however, is not a disagreement since Owen has had to assume in his hypothesis that *W* was complementary to r_2 to produce the imperfect-black color of the seed coat.

With the hypothesis presented in this paper the results obtained by crossing the plant from the Ito San variety with Selection No. 3 (Owen (*5*)) can be explained without assuming an allelomorphic series

at the R_2 locus. Also, a particular color can be designated by the same formula regardless of the variety in which it appears.

It may be well to mention that the author has crossed the Ito San variety with the varieties Black Eyebrow and Manchu, both of which contain the factor R_2. In neither case was there any evidence of a segregation for R_2, and, therefore, no indication of an allelomorphic series at the R_2 locus.

SUMMARY

Data for the F_2 and F_3 generations are presented to show the inheritance of yellow, black, imperfect-black, light-brown, reddish-brown, and buff colors in the seed coat of soybeans.

Two complementary factors R_1 and R_2 cause the development of black pigment. With R_1r_2 the color is a dilute or imperfect-black, with r_1R_2 the pigment color is light-brown, and with r_1r_2 the color is buff.

A series of allelomorphs, R_1r_1 and $r_1{}^o$, has been found to exist at the R_1 locus. R_1 with R_2 produces black pigment, r_1 with R_2 light-brown, and $r_1{}^o$ with R_2 a reddish-brown color.

Two pigment restriction factors I and I^k form an allelomorphic series with i. I restricts any of the pigment colors to the hilum or regions around the hilum. I^k restricts pigments in such a way as to give the eyebrow pattern. The factor i has no inhibiting effect and when present in the homozygous condition the seeds are self-colored.

The factor R_2, if not the same as T, the factor for tawny pubescence, is closely linked with T. No crossing over between the two factors has been observed in these studies.

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THE NORMAL LIMITS OF VARIATION OF THE METHYLENE-BLUE REDUCTION TEST [1]

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INTRODUCTION

The methylene-blue reduction or reductase test as a means of judging the sanitary quality of milk has met with great popularity in this country. This method of analysis has the advantages of being very simple to operate, easy to interpret, and requires very little expense for materials. Many of the small milk plants not equipped with elaborate facilities necessary to make agar-plate counts are using the methylene-blue reduction test as a means of detecting undesirable sources of milk supply. In many cities it has been adopted as a part of the system of regulatory control of the milk supply. Milk inspectors in the larger cities frequently use this test for general survey work and supplement it with other more elaborate tests.

The wide use of the test under many conditions has led to many modifications of the procedure, which for the most part are designed to render it even more simple and practical. Many of those who use this test are interested only in a rather rough approximation of quality; hence, extreme accuracy in measuring the ingredients for the test and strict aseptic precautions have not been deemed essential. In some dairy plants the 10 c. c. of milk used in the test is measured by means of a dipper instead of a pipette; in others, graduated test tubes are used. Instead of using bacteriologically sterile glassware, as is necessary in the plate count, the test tubes are placed in boiling water a few minutes, and the measuring dippers when not in use are kept either in hot water or in a disinfectant solution. Although these practices are admittedly inaccurate, the errors are commonly regarded as of no practical significance.

The preparation of the methylene-blue solution has been simplified by the use of specially prepared tablets that contain a standard amount of the dye, thus eliminating the necessity for expensive chemical balances to weigh small quantities of the powder. One tablet is dissolved in 200 c. c. of water which has been partially sterilized by boiling for a few minutes.

The present investigation was designed primarily to determine the expected limits of normal variation of the methylene-blue test in order to afford more concrete evidence of its accuracy and reliability. If a sample of milk reduces the methylene blue in two and one-half hours, may a second determination be expected to give the same result? If variations are observed, are they likely to be a few minutes or one or two hours? In addition to answering these questions an effort was made to measure the effect of the modifications of technic that have been adopted as a means of simplifying the test.

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If a proposed modification simplifies the test at the expense of accuracy, it is not to be recommended unless it can be shown that the variation introduced by the simplified technic is insufficient in magnitude to be of practical significance.

REVIEW OF LITERATURE

Thornton and Hastings [2] found that the methylene-blue reduction was dependent upon the dissolved oxygen and that a concentration of 1:200,000 of methylene blue gave approximately the same results as 1:100,000. They concluded that extreme accuracy in measuring the sample is not necessary if reduction occurs in less than five and one-half hours. They, like Prucha and Ambrose, as reported by Mumford,[3] found only a very general correlation between the reductase test and the plate count.

A report from the Wisconsin station [4] indicates that there is considerable variability in the methylene blue from different sources. The prepared tablets were found to be sufficiently uniform for practical purposes, and the adoption of these was recommended.

Troy [5] compared the methylene-blue reduction and direct microscopic methods of analysis and reported a correlation of 86.2 per cent ($r=0.862$).

Results obtained by Martin [6] at the Pennsylvania Agricultural Experiment Station indicate a close agreement of the methylene-blue reduction test and the sediment test for grading milk. Thus, of the samples placed in the first grade by the sediment test, 82 per cent were similarly classified by the methylene-blue reduction test.

PLAN OF EXPERIMENT

In order to study the normal variation which may be expected with the reductase test, relatively large numbers of determinations were made from the same sample of milk. Similarly, in studying the effect of proposed modifications, a relatively large number of parallel tests were made with the same sample of milk, two or more methods being used. Extreme care was exercised to control all variables except those under observation.

In order to prevent a marked change in the number of microbes in the milk during the time required to set up the experiment, the following procedure was adopted in all cases. The milk to be used in the experiment was first chilled in iced water for 20 to 30 minutes. The chilled milk was agitated while the requisite number of 10 c. c. portions were being placed in the tubes. Only 20 tubes were filled at a time, and the basket of filled tubes was immediately replaced in the the iced water. As soon as the required number of tubes were thus prepared, the methylene blue was added to each tube. The basket of tubes was immediately placed in a water bath at 37° C. and the time recorded. By setting up a few tubes at a time and by keeping

[2] THORNTON, H. R., and HASTINGS, E. G. SOME ASPECTS OF THE METHYLENE-BLUE REDUCTION TEST OF MILK. Jour. 13: 62–63. 1927.

[3] MUMFORD, H. M. A YEAR'S PROGRESS IN SOLVING SOME FARM PROBLEMS IN ILLINOIS. Ill. Agr. Expt. Sta. Ann. Rpt. 40: 158–159. 1927.

[4] RUSSELL, H. L., MORRISON, F. B., and EBLING, W. H. NEW PAGES IN FARM PROGRESS, METHYLENE BLUE REDUCTION TEST STANDARDS. Wis. Agr. Expt. Sta. Ann. Rpt. 1923–24: 71–72. 1925. (Bul. 373.)

[5] TROY, H. C. A COMPARISON OF THE METHYLENE-BLUE TEST AND THE DIRECT MICROSCOPIC COUNT IN GRADING MILK AT MILK PLANTS. Jour. Dairy Sci. 8: 282–285. 1925.

[6] MARTIN, W. H. JUDGING THE QUALITY OF MILK BY THE METHYLENE-BLUE REDUCTION TEST, SEDIMENT TEST, AND BACTERIA COUNT (PLATE METHOD). Penn. Agr. Expt. Sta. Ann. Rpt. 38: 23. 1925 (Bul. 196.)

the milk in iced water until the methylene blue was added the variation in the reduction time caused by bacterial growth was held to a minimum. In all but three experiments the elapsed time between placing the first and last basket of tubes in the 37° C. water bath was about 30 minutes. In the three exceptions the average reduction time of the milk was so short that it was necessary to complete the reduction test on one basket of tubes before adding the methylene blue to another.

The variation in the reduction time for the replicate tests on the same sample of milk was calculated and expressed in most cases as a coefficient of variability. Other statistical devices were resorted to as occasion seemed to demand.

RESULTS

In Table 1 are recorded the results of 1,774 tests on 19 samples of milk. From 75 to 100 tests were made on each sample; in some cases 20 to 50 tests were made by two or more methods, using the same sample of milk.

TABLE 1.—*Variations of the methylene-blue reduction test in the examination of various samples of milk*

Experiment No.	Tests	Reduction, time			Coefficient of variability	3.2 times the probable error	Plate count per cubic centimeter	Modification of methods in reductase test
		Extremes		Average				
		Low	High					
	Number	*Minutes*	*Minutes*	*Minutes*	*Per cent*	*Minutes*		
1	80	25	39	29	8.9	6	400,000	10 c. c. pipette.
2a	50	31	44	36	10.5	8	300,000	10 c. c. cup.
2b	50	42	55	52	8.9	10	300,000	Graduated test tube.
3	75	28	50	40	13.0	11	5,000,000	Do.
4	80	79	106	93	8.5	17	270,000	10 c. c. pipette.
5a	20	71	74	72	1.4	2	4,000,000	0.5 c. c. methylene blue.
5b	20	98	104	102	2.0	4	4,000,000	0.8 c. c. methylene blue.
5c	20	113	127	119	4.4	11	4,000,000	1 c. c. methylene blue.
5d	20	125	133	129	2.0	6	4,000,000	1.2 c. c. methylene blue.
5e	20	132	139	135	1.9	5	4,000,000	1.5 c. c. methylene blue.
6a	50	105	115	106	1.6	4	3,000,000	Dye solution prepared from tablet.
6b	50	107	112	108	1.8	4	3,000,000	Dye solution prepared from powder.
7	80	135	171	151	5.7	19	25,000	10 c. c. pipette.
8	86	157	164	161	1.2	4	----------	Do.
9a	50	201	223	209	2.3	10	200,000	10 c. c. cup.
9b	48	165	242	219	7.0	33	200,000	10 c. c. pipette.
9c	50	226	241	232	1.8	9	200,000	Graduated test tube.
10a	52	211	254	241	3.3	7	----------	Glassware in boiling water 15 minutes.
10b	51	245	269	257	1.8	4	----------	Glassware in 20 pounds steam 30 minutes.
11	100	233	286	252	10.9	23	----------	10 c. c. cup.
12	80	371	406	383	3.4	28	30,000	10 c. c. pipette.
13	60	366	407	390	2.6	22	11,000	Do.
14a	20	364	386	374	1.6	13	19,000	0.5 c. c. methylene blue.
14b	20	390	404	397	1.1	9	19,000	0.8 c. c. methylene blue.
14c	20	399	421	408	1.5	13	19,000	1 c. c. methylene blue.
14d	20	408	428	419	1.8	16	19,000	1.2 c. c. methylene blue.
14e	20	413	439	426	1.6	15	19,000	1.5 c. c. methylene blue.
15	82	381	440	419	3.1	28	37,000	10 c. c. pipette.
16a	50	384	448	420	4.9	44	14,000	Dye solution prepared from powder.
16b	50	393	474	433	5.0	46	14,000	Dye solution prepared from tablet.
17	100	404	508	462	4.3	43	60,000	10 c. c. cup.
18	100	409	561	484	6.8	71	8,000	Do.
19	100	319	550	490	6.9	72	28,000	Graduated test tube.

The data include the extremes, the average reduction time, the coefficient of variability, the probable error multiplied by the factor 3.2, and the plate count for each sample of milk. Any modifications of the usual procedure for the test are designated in the last column on the right. Where two or more modifications were applied to

single sample of milk, the subdivisions of the experiment are designated by a number and a letter (i. e., 2a, 2b, etc.).

Since the effect of the various modifications is brought out in a subsequent discussion, Table 1 is intended to show only the general range and degree of variability of the methylene-blue reduction test and its relation to the plate count. The experiments are arranged approximately in the order of their average reduction time. The probable error multiplied by the factor 3.2, as given in column 7, establishes the limits within which there is a 30 to 1 chance that a subsequent determination will fall. The results with sample No. 1, for example, indicate a practical certainty (30 to 1 chance) that the reduction time of a single test on this milk will not be in error more than six minutes.

A general survey of the figures in the column showing the coefficient of variability reveals that in 14 cases this value was 2 per cent or less. In general these results indicate a very satisfactory degree of uniformity.

With a few exceptions the samples requiring from one and one-half to seven hours for reduction (5e–14) show relatively lower degrees of variation than those requiring more or less time for reduction. In other words, when the reduction time is very short or very long, greater variation in results may be expected.

Some idea of the reliability of the reduction test may be secured by a study of the extremes of variation. Thus, there are only two instances (9b and 11) among the samples requiring less than 7 hours for reduction in which the greatest variation was more than 30 minutes from the average reduction time. In other words, with but two exceptions, if a single test had been accepted on any of the first 14 samples of milk, the result would not have been more than 30 minutes in error. In 6 of the 14 samples requiring less than 7 hours for reduction, the widest deviation from the average was 10 minutes. In the samples requiring from 7 to 8 hours for reduction the deviation from the average ranged from 40 to 95 minutes.

A more concrete idea of the reliability of this test may be obtained from the column showing the values for 3.2 times the probable error. With one exception (9b), one could be reasonably certain that the result of a single test on any one of the first 15 samples would not be more than 30 minutes in error. In a similar way, the values for the samples requiring from 7 to 8 hours for reduction range from approximately 40 to 95 minutes.

The results of these experiments demonstrate clearly the reliability of the methylene-blue reduction test. If there is a 30 to 1 chance that the results of a single test are not more than 10 to 30 minutes in error, there is little likelihood that the sanitary quality of a sample of milk will be grossly misjudged when the results are interpreted.

In calculating the correlation between the reduction time and the plate count the results of samples 5a, b, d, e, and 14a, b, d, e, were eliminated because of the obvious effect of adding varying quantities of methylene blue. A general survey and comparison of the figures in Table 1 showing plate counts and reduction times indicate a fair, though by no means perfect, correlation between the two tests. The coefficient of correlation was found to be -0.82 ± 0.48. The agreement may be considered good and corroborates the foregoing conclusion as to the reliability of the methylene-blue test.

EFFECT OF MODIFICATIONS OF THE REDUCTASE TEST

By the elimination of many technicalities the methylene-blue reduction test has been very successfully adapted for field conditions and for use in small dairy plants. In the following tables data are presented to show the effect of some of the modifications on the reliability of the test.

BACTERIOLOGICALLY STERILE GLASSWARE

In Table 2 are presented the results of 103 tests on the same sample of milk, in 52 of which the test tubes and pipettes were subjected to boiling water for 15 minutes, and in the remaining 51 tests bacteriologically sterile glassware was used. The average reduction time for the tests in which bacteriologically sterile glassware was used was 257 minutes, as compared with 241 minutes for the other. The question immediately arises whether the difference of 16 minutes is within the limits of normal variation of the test.

In Table 2 and succeeding tables the actual difference between two average reduction times has been divided by the probable error of the difference; if the quotient exceeds 3.2, it is fairly safe to conclude that the modification of the method introduces a measurable variation. Table 2 shows that when the actual difference of 16 minutes is divided by the probable error of the difference a quotient of 6.4 is obtained. This indicates that the use of glassware which has been boiled 15 minutes introduced a measurable error into the results for this sample of milk. It does not necessarily follow, however, that statistical significance and practical significance are coincident.

TABLE 2.—*The effect of using bacteriologically sterile glassware and "practically sterile" glassware on the results of the methylene-blue reduction test in the examination of milk*

Sample No.	Tests	Method of sterilizing glassware	Average reduction time	Difference divided by the probable error of the difference
	Number		*Minutes*	
19a	52	Boiling water, 15 minutes	241±2.2	6.4
19b	51	Autoclave, 20 pounds pressure, 30 minutes	257±1.2	

METHODS OF MEASURING MILK

Table 3 shows the results of experiments on two samples of milk in which various methods of measuring the sample were compared. In the first sample the tests measured by means of graduated test tubes and with a 10 c. c. cup gave an average reduction time of 52 and 36 minutes, respectively. The difference is four times the probable error of the difference.

The second sample was measured by three methods and resulted in an average reduction time of 232 minutes for the samples measured in a graduated test tube, 209 minutes for the 10 c. c. cup method, and 219 minutes for those measured with a 10 c. c. pipette. Here again the result with the cup method deviates from the graduated test tube method by an amount equal to 5.4 times the probable error.

The difference between the pipette and the graduated test tube methods (13 minutes) is but slightly more than the probable error of the difference, and hence may be regarded as of no significance.

TABLE 3.—*The effect of various methods of measuring the 10 c. c. sample of milk used on the results of the methylene-blue reduction test*

Sample No.	Plate count per cubic centimeter	Tests	Methods of measuring milk	Average reduction time	Difference divided by the probable error of the difference when using—	
					10 c. c. cup	10 c. c. pipette
		Number		*Minutes*		
1	300,000	50	Graduated test tube	52	4.0	
		50	10 c. c. cup	36		
2	200,000	50	Graduated test tube	232	5.4	1.2
		50	10 c. c. cup	209		.9
		48	10 c. c. pipette	219		

STANDARD METHYLENE-BLUE TABLETS

Two solutions of methylene blue were prepared: (1) By dissolving a standard methylene-blue tablet in 200 c. c. of sterile water; and (2) by making a 1 per cent aqueous solution from a carefully weighed sample of the powder and adding 1 c. c. of this solution to 199 c. c. of sterile water. For convenience the latter was called the laboratory solution and the former the tablet solution. These two solutions were tried in parallel experiments on two samples of milk, one of which had a low bacterial count (14,000 per cubic centimeter) and the other a high bacterial count (3,000,000 per cubic centimeter). Table 4 shows that the difference between the average reduction times for the two solutions was only 13 minutes in the milk with low bacterial count and only 2 minutes in the milk containing 3,000,000 bacteria per cubic centimeter. These differences are not significant.

TABLE 4.—*A comparison between the use of a methylene-blue solution prepared in the laboratory and one prepared from a commercial tablet in making the reduction test on milk*

Sample No.	Plate count per cubic centimeter	Tests	Kind of methylene-blue solution used	Average reduction time	Difference divided by the probable error of the difference
		Number		*Minutes*	
1	14,000	50	Laboratory solution	420±13.8	0.6
		50	From tablet	433±14.5	
2	3,000,000	50	Laboratory solution	108±1.2	1.2
		50	From tablet	106±1.2	

INACCURACIES IN MEASURING THE DYE SOLUTION

The experiments reported in Table 5 illustrate the effect of adding varying quantities of the methylene-blue solution to the milk. The purpose was to determine the effect of careless measuring or the use of inaccurate pipettes. Two samples of milk were used, one of which gave a plate count of 4,000,000 and the other 19,000 per cubic centimeter. One hundred tests were made on each sample, using varying

amounts of the dye solution in each of five series of 20 tests each. To each tube of a series 0.5, 0.8, 1.0, 1.2, or 1.5 c. c. of methylene-blue solution were added. With both samples the reduction time increased directly as the quantity of the dye solution was increased.

In measuring 1 c. c. of methylene blue it is very unlikely that an error of 0.5 c. c. would result from careless technic or the use of a faulty pipette; however, an error of 0.2 c. c. might easily occur from either of these sources.

The results of these experiments indicate very clearly that the amount of methylene blue used has a direct bearing on the time required for reduction. Gross error in measuring the dye solution will introduce a noticeable effect upon the results of the test and should not be tolerated. Some of the commercial outfits for making this test are equipped with an eye dropper for measuring the dye solution. The relatively large bore of the short tube and the correspondingly wide meniscus are not conducive to accurate measuring. It is doubtful if these should be used.

Attention is called to the fact that the addition of less than 1 c. c. of dye solution introduced greater variation than when more than the standard amount was used; also that the milk with the shorter reduction time (sample 1) was more noticeably affected by errors in measuring the dye than the other sample.

The maximum deviation in the average reduction time resulting from varying the amount of dye solution was 63 and 52 minutes, respectively, for samples 1 and 2. Although these values are probably not of sufficient magnitude to seriously impair interpretation of the test, nevertheless they indicate that care should be exercised in measuring the methylene-blue solution.

TABLE 5.—*The effect of adding varying quantities of methylene blue on the results of the reduction test on milk*

Sample No.	Plate count per cubic centimeter	Tests	Amount of methylene-blue solution used	Average reduction time	Difference divided by the probable error of the difference
		Number	*c. c.*	*Minutes*	
1	4,000,000	20	0.5	72±0.7	13.1
		20	.8	102±1.4	4.5
		20	1.0	119±3.5	
		20	1.2	129±1.8	2.5
		20	1.5	135±1.7	4.1
2	19,000	20	.5	374±4.1	5.9
		20	.8	397±2.9	2.2
		20	1.0	408±4.0	
		20	1.2	419±5.1	1.7
		20	1.5	426±4.7	2.9

SUMMARY AND CONCLUSIONS

The data presented in this paper confirm the general belief among laboratory technicians that methylene-blue reduction tests are characterized by a low degree of variability. When a large number of tests (75–100) were made on each of 19 samples of milk, it was found that the maximum and minimum reduction time for each sample were relatively close together. If the 19 samples of milk had been classified according to the method usually employed in this test, there would

have been 8 in class 1 (reduction time more than 330 minutes), 5 in class 2 (reduction time between 120 and 330 minutes), and 6 in class 3 (reduction time between 20 and 120 minutes).

The values for 3.2 times the probable error for the class 3 samples ranged from ±2 to ±17 minutes. One could be reasonably certain that the results of a single test on most of these class 3 samples would not be more than 10 to 15 minutes in error. The limits of a similar degree of reliability for the milk in class 2 were approximately ±30 minutes, although several samples were far less variable and one was more variable than this would indicate. Likewise, one could be reasonably certain that the reduction time of class 1 milk would not be more than ±30 minutes in error, providing it did not require more than seven hours for reduction to take place. Of the class 1 samples requiring approximately 7 and 8 hours for reduction, the limits of certainty of the test were 45 and 70 minutes, respectively.

The data also show that the various modifications designed to simplify the test did not seriously impair its accuracy. For example, the difference in the average reduction time for tests in which the sample was measured with a 10 c. c. pipette and a 10 c. c. standard cup was 10 ± 10 minutes; when graduated test tubes were used instead of pipettes the average reduction time was increased 13 ± 10 minutes. The use of glassware that had been only partially sterilized by boiling lowered the reduction time 16 ± 2.5. Parallel tests using a methylene-blue solution prepared from a tablet and one prepared from the powdered dye gave a difference of 13 ± 20 minutes on milk requiring 7 hours for reduction, and 2 ± 1.7 minutes on another sample of milk in which the reduction time was a little less than 2 hours.

The results of experiments in which varying amounts of dye solution were added indicate that the reduction time is directly related to the amount of dye added. When the inaccuracies of measuring the dye were within the limits of 1 ± 0.2 c. c., an error of 10 to 15 minutes was introduced in the results. However, errors of 0.5 c. c. in measuring the methylene-blue solution introduced variations of 30 to 47 minutes.

The direct relationship between reduction time and the amount of dye solution added indicates that gross errors in measuring would introduce variations of sufficient magnitude to impair the accuracy of the test. Since there is no particular end gained by careless measuring or the use of inaccurate measuring devices, and since the amount of methylene blue added has such a direct bearing on the results obtained, it is advisable to use care in measuring the dye.

When the data are treated statistically, it is found that the limits of normal variation are so close together that even errors of a relatively few minutes are well outside the expected limits of error of the reductase test. Even though an error of 10 to 15 minutes may be significant from a statistical point of view, variations of this magnitude are well within the demands for accuracy in the routine analysis of milk. Many of the modifications have increased the practicability of the test and have made it applicable for use in small plants. The results of these experiments indicate that the simplified methods of making the reductase test which have so greatly enlarged its field of adaptability do not seriously affect the interpretative value of the test.

SEX DIFFERENCES IN THE NORMAL GROWTH RATE OF CHICKS[1]

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There is frequent need for normal growth standards in certain phases of chick nutrition work. Among such needs is a standard for the reduction of the population of a lot or group to a single standard for comparison with other lots identically treated but varying in the distribution of the sexes within the lot. Several expedients have been used to compensate for this variation. If the lots chance to be made up of equal numbers of either sex, comparisons may be made directly, but in the case of experimental work with very young chicks, since the sexes can not be differentiated at hatching time, the random selection of the group can not insure equality of numbers of either sex chosen.

Three ways of correcting for the resulting discrepancy in the numbers suggest themselves. The growth data of a group may be corrected to a basis of all males, to a basis of all females, or to the basis of an even division of the sexes. The choice of the method to be used will be a matter of individual preference. The element lacking is the conversion factor. In the interpretation of some work done at the Nebraska station it was thought desirable to attempt the determination of such a factor for Single Comb White Leghorn chicks. The growth records of several hundred chicks from 1 week to 9 weeks of age were available as a result of numerous projects involving them as subjects.

Some of these experiments were designed to produce nutritional failure with death or subnormal growth as the direct result. Such birds were obviously unsuited to the determination of a normal conversion factor, so of course the data on such lots were not included. It was, however, possible to fix an arbitrary standard of growth, the one determined upon being an average weight of 550 gm. for the lot at the age of 9 weeks, before including that lot in the tabulation. In this way lots of birds which had, on the whole, made normal growth or better were used, and the records of lots making subnormal growth were excluded from the calculations.

Thus while it is true that all the chicks were not raised on the same ration, they were all raised on rations which promoted satisfactory growth. The brooding conditions under which the different lots of chicks were raised were good, as is indicated by low mortality in all lots chosen. The chicks were raised in a steam-heated brooder house divided into several runs 4 feet wide and 13 feet long. From 25 to 35 chicks were brooded in each of these pens. Some, but not all, of the lots had access to screened inclosures 4 feet square on the south side of the brooder house when exposure to direct sunshine was desired. The floor was of concrete with pine shavings serving as litter. Electrically heated hovers were used for temperature control. The all-mash method of feeding was employed, no scratch feed being given. Tap water was before the birds at all times.

[1] Received for publication Nov. 22, 1929; issued May, 1930. Contribution from the Departments of Agricultural Chemistry and Poultry Husbandry. Published with the permission of the director of the Nebraska Agricultural Experiment Station as paper No. 84, Journal Series. This paper was read before the division of biological chemistry, American Chemical Society, Minneapolis, Minn., Sept. 10, 1929.

Hendricks, Lee, and Titus [2] suggested that the slightly lower growth secured in their work for the first 7 to 10 weeks might have been due to rearing the chicks on concrete floors and under conditions of partial confinement. In the work here reported the chicks were also reared on concrete floors. They had no access to soil in the yards, and each lot had a maximum floor space of 68 square feet. Since they made satisfactory growth under these conditions, it would seem that other factors than close confinement to concrete yards must have been responsible for the lower growth reported by the above writers.

When the selections from the record were complete it was found that growth data on 397 males and 403 females were available for the purposes of the study. The chicks were leg-banded at hatching time. When it became possible to distinguish the sex of the bird this fact was noted on the record, so that the individual sex and weight records were complete. Individual weighing were made at 1, 3, 5, 7, and 9 weeks of age. These data were then analyzed statistically at the five ages for each sex, and the mean, the probable error of the mean, the standard deviation together with its probable error, the probable error of the weight of a single chick, and the coefficient of variability and its probable error were all found by the method outlined by Davenport.[3] The results of this analysis are shown in Table 1.

TABLE 1.—*Mean weights of chicks at different ages*

FEMALES

Age in weeks	Mean weight	Standard deviation	Probable error per single chick	Coefficient of variability
	Grams			*Per cent*
1	57.5±0.28	8.47±0.20	5.71	14.72±0.36
3	128.7± .80	23.89± .57	16.11	18.56± .46
5	242.5±1.64	48.90±1.16	33.02	20.18± .59
7	380.1±2.57	76.56±1.82	51.64	20.14± .59
9	532.0±3.43	102.00±2.40	68.79	19.17± .47

MALES

1	60.4±0.31	9.09±0.22	6.13	15.05±0.37
3	137.5± .99	29.10± .70	19.63	21.16± .52
5	259.5±1.95	57.73±1.38	38.94	22.25± .56
7	418.7±2.96	87.42±2.09	58.96	20.87± .52
9	606.4±4.00	118.16±2.83	79.73	19.49± .48

The mean female weight can now be compared with the mean male weight, and the differences between the means at the five ages, together with the probable error of these differences, can be calculated, as has been done in Table 2. The ratio of these differences to their probable errors, then, indicates the significance of the differences. It will be noted that when dealing with 400 males and 400 females there is a significant difference between the mean male and mean female weights from the first weighing to the last. This bears out the conclusion of Jull,[4] who stated that much larger numbers of

[2] HENDRICKS, W. A., LEE, A. R., and TITUS, H. W. EARLY GROWTH OF WHITE LEGHORNS. Poultry Sci. 8: 315-327. 1929.

[3] DAVENPORT, E. PRINCIPLES OF BREEDING; A TREATISE ON THREMMATOLOGY OR THE PRINCIPLES AND PRACTICES INVOLVED IN THE ECONOMIC IMPROVEMENT OF DOMESTICATED ANIMALS AND PLANTS. (With appendix by H. L. Rietz.) 727 p., illus. Boston, New York, [etc.]. 1907.

[4] JULL, M. A. DIFFERENTIAL SEX GROWTH CURVES IN BARRED PLYMOUTH CHICKS. Sci. Agr. 4: 58-65, illus. 1923.

birds than he used in his study would have to be employed to show a significant difference in mean male and female weight before the eighth week of life. Two other columns are included in Table 2, the first comparing the female weight as a percentage of the male weight, the second showing the ratio of the male to the female weight.

TABLE 2.—*Comparison of the mean weights of male and female chicks at different ages*

Age in weeks	Difference between means	Ratio of difference to its probable error	Mean female weight as per cent of mean male weight	Mean male weight divided by mean female weight
	Grams			
1	2.9±0.42	6.9	95.21	1.051
3	8.8±1.28	6.9	93.61	1.069
5	17.0±2.55	6.7	93.44	1.070
7	38.6±3.92	9.8	90.78	1.102
9	74.4±5.28	14.1	87.72	1.140

While there is a significant difference between the mean male and female weights at 1 week of age, it can be readily appreciated that the sex of the week-old chick can not be inferred from its weight, for the magnitude of the probable error of the weight of a single chick, or of the coefficient of variability, precludes the possibility of such an inference.

The point to be observed is that the mean weight of a group of chicks at 9 weeks of age is influenced by the number of males in proportion to the number of females. If the distribution between the sexes is not equal, discrepancies will arise when lots are compared on the basis of the mean weight of the lots without making a correction in the proper sense. It would seem feasible to make such a correction by using the factor of the ratio of male to female weight as found in a study of the growth rates of 400 individuals of each sex. As noted in Table 2, this ratio is, at 9 weeks of age, female weight times 1.140 equals male weight. This is the procedure followed in correcting the weight of the females of a lot to the basis of the weight of all males. It can be applied individually to convert the weight of each female to a male basis, or the average female weight can be converted to a male basis, and from this value, together with the actual weights of the males involved, the whole lot can be brought over to the basis of all males. That the distribution of sexes in the lot is a factor is shown by Table 3.

TABLE 3.—*Effect of unequal division of sexes on weight averages in flocks of 40 chicks*

Males	Females	Average weight of 40 chicks	Males	Females	Average weight of 40 chicks
Number	*Number*	*Grams*	*Number*	*Number*	*Grams*
0	40	532	25	15	578
5	35	541	30	10	588
10	30	550	35	5	597
15	25	560	40	0	606
20	20	569			

Take a lot of 40 birds and assume that the mean weight of the males is 606 gm. and that of the females 532 gm. Varying the number of males in this hypothetical lot from 0 to 40, while the number of females is varied conversely, will bring out the discrepancies that arise from a failure to take this distribution into account.

If the average weight is calculated upon the total weight of all the males and females, divided by 40, the above table is obtained. This illustrates the fact that the average or mean weight of the lot would depend on the ratio of male to female birds in the lot. However, by the use of the factors found in Table 2, it is possible to compare a lot with one proportion of males to a lot having another proportion. By the use of the factor the lot may be converted to the basis of all males, in which case the mean weight would be 606 gm., all females, in which case the mean weight would be 532 gm., or half males and half females, in which case the mean weight would be 569 gm. Direct comparison is thus obtained regardless of the basis selected. If the mean weight of each lot is to be used as one of the criteria the lots must be brought to a uniform basis before comparison is attempted.

SUMMARY

The growth data of 397 male and 403 female Single Comb White Leghorn chicks from 1 to 9 weeks of age have been subjected to a statistical examination. Differences in the mean weight of male and female chicks are significant at 1 week of age, and the significance increases thereafter.

A factor is suggested for use in bringing lots with different numbers of male and female chicks to a common basis for the purpose of comparison.

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